Mechanisms Underlying Dementia and Stroke

an immunity and inflammation perspective

Lana Fani

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Mechanismen onderliggend aan dementie en hersenberoertes
vanuit het perspectief van het immuunsysteem en inflammatie

Mechanisms Underlying Dementia and Stroke
an immunity and inflammation perspective

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Voor mijn lieve ouders

Stairwells

Thousands of scientists
are trying to comprehend
the billions of connections
that relentlessly extend

through different layers
of distinct cells
passing electrical signals
like pouring water from stairwells.

The source of our behavior,
our thinking,
our entire mind.
A super construction, of which the blueprint we must find

to cure people who forget,
cannot speak,
are paralysed.
Their independence severely compromised.

L. Fani

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*Both authors contributed equally to this study

PROLOGUE

I remember well the image of a real brain during a neurosurgical procedure I attended as a medical student. It was pulsating calmly to the rhythm of the heart, yet looking so vulnerable as it was exposed when the piece of skull was removed. A little scratch to this soft pink organ could have devastating consequences as I learned. I was intrigued by this organ. How could it be so powerful, controlling our entire body, yet be so prone to damage? And damage could occur in a vast variety of forms: from tumours to vascular damage, to trauma. Moreover, in contrast to for example trauma, where it is clear where the damage comes from, we often do not know why damage to the brain occurs in the first place. Why do some people develop brain tumours? Like my mom, who fortunately recovered. Why do others have extensive vessel disease in their brain? Why does the brain shrink so fast in some people? All these phenomena have devastating consequences. I fell in love with the brain, with its mystery, its complexity and its weaknesses.

The cover of my thesis depicts the grey matter of a normal brain with 'resting' microglia cells stained with Rio Hortega`s silver carbonate method. Microglia are the guardians that react to potential danger to the brain. But the question is, could they also become traitors? While my thesis does not specifically focus on microglia, it does attempt to answer this question, among others, by viewing inflammation or the immune system as a potential mechanism that may cause dementia or stroke.

Chapter 1
General Introduction

The brain is a fascinating and unique organ which orchestrates our entire behaviour. The earliest reference to the brain anywhere in human records is in an ancient Egyptian medical treatise called the Edwin Smith Surgical Papyrus (**Figure 1**).[1] Since then, our understanding of the brain has come a long way, however many mysteries still remain.[2] One of those mysteries is the neural basis underlying psychiatric and neurological disease. With an ageing population projected to include 152 million people with dementia by 2050[3], along with the rising global burden of disability attributed to stroke[4], these two diseases play a key role in the worldwide health burden.[5]

Figure 1. Hieroglyphic for "Brain". *From Principles of Neural Science.*

DEMENTIA: SENILE INSANITY

The word dementia derives from the Latin root *demens*, which means being out of one's mind. The term "dementia" has been used since the 13th century, but its mention in the medical community was only reported in the 18th century.[6] Dementia was recognized as having two types of brain changes. In some of these brains, numerous healed infarcts could be seen, which were clearly the result of arteriosclerosis; in the others, the only evident change was a generalized atrophy as a consequence of aging.[7] Alzheimer wrote his first major article in 1899 on the nature of senile insanity or dementia in which he maintained that those cases of atrophy without recognizable infarcts also were due to vascular disease, but that this involved arterioles rather than arteries, so the infarcts were microscopic.[8] By 1906, when Alzheimer's disease (AD) was discovered, Alzheimer had moved to Munich where he became the chief of pathology in the lavish new institute of brain research headed by Emil Kraepelin, the foremost psychiatrist in the world.[9] Kraepelin was an organist, believing that all mental disease was due to some alteration of the brain as an organ, opposing the Freudian concept of psychoanalysis in which mental disease was due to a conflict between the consciousness and subconscious mind.[10] Kraepelin lay the foundation of the modern classification system for mental disorders, and remarkable similarities remain in the now widely used Diagnostic and Statistical Manual of Mental Disorders (DSM), of which the latest, fifth edition appeared in 2013.

STROKE: APOPLEXIA

As far as 2,500 years ago the term *apoplexia* has been found in writings, meaning "struck down with violence", describing a disorder in which "a person suddenly falls, without consciousness or motion, retaining pulse or respiration".[11] This characteristic picture was well known to the ancient Greeks, and it was Hippocrates who was responsible for the first recorded appearance of the term "apoplexy".[12] During The Renaissance, the promotion of human dissection allowed society to use autopsy for forensic, health and scientific purposes, paving the way for modern medicine.[13] During this modern era from the 17th century onwards, "apoplexy" began to lose its unitary (umbrella) meaning,[14] marking the start of studies on vascular diseases of the brain or "cerebrovascular disease(s)"[11], after which Cole (1689) first used the term "stroke".[15] This term then appeared only much later in the ICD-9 in 1968.[16] The definition of the concept by the World Health Organization (WHO) appeared soon after in 1971 and 1980, and more recently, a new definition was proposed by the American Heart Association-American Stroke Association (AHA-ASA) in 2013, incorporating clinical and tissue criteria.[17] Stroke is nowadays widely recognized as the appearance of acute focal neurological symptoms or signs that result from diseases involving cerebral blood vessels.[18]

WHY STUDYING DEMENTIA AND STROKE?

The etiological similarity between dementia and stroke was already suggested in Alzheimer's first major article on dementia mentioned earlier, describing that those cases of atrophy without recognizable infarcts were due to microscopic infarcts[8]. This idea was further supported by Walter Alvarez, a gastroenterologist, whose interest in AD was stimulated by the presence of this condition in his father-in-law.[7] The episodic aggravation of his father-in-law's condition led him to the conclusion that these changes were the results of small episodes of brain damage, ultimately known as little strokes.[19] Indeed, it is now recognized that all major dementias have a vascular component, ranging from 61% in frontotemporal dementia to 80% in AD.[20] In fact, the presence of a vascular component doubles the probability that the neurodegenerative pathology will have manifested as dementia later in life.[21] At the same time, not all dementias can be prevented by preventing stroke and there are growing genetic, transcriptomic and proteomic data pointing to the complexity of AD pathogenesis.[22] It is thus essential to gain a better understanding of the underlying pathophysiology that may lead to both these diseases.[23]

DEMENTIA AND STROKE: AN IMMUNITY AND INFLAMMATION PERSPECTIVE

When Alois Alzheimer first described the histopathology of AD, he noted, "The glia have developed numerous fibers".[24] It was only in the late 1980s that scientists began to study this feature.[25] It included the involvement of the immune system in AD, and was shown by the attachment of complement proteins to diseased tissue, and by the activation of cells associated with the immune system.[26,27] Over the last 10 years, this field of research saw a steep increase[25] after the discovery of AD-associated variants in the triggering receptor expressed on myeloid cell 2 (*TREM2*) gene that is only expressed on immune cells of myeloid origin. Further support came from a large-scale genome-wide association study that identified multiple additional immune risk factors[28], but to date, it is still not clear whether immunity and inflammation in fact lead to AD or become only present in a later stage, when the disease already started. In order to shed some light on this issue, **chapter 2** explores the effects of the immune system on the risk of dementia using population-based data. **Chapter 2.1** considers immune cells in relation to dementia risk. These immune cells consist of granulocyte and platelet count as measures of the innate immune system and lymphocyte count as measure of the adaptive immune system. In the field of cancer research it has been suggested that taking the ratio of these innate and adaptive immune cells results in new white blood-cell based inflammatory indices as markers of low-grade inflammation.[29] These are the neutrophil-to-lymphocyte ratio (NLR), the platelet-to-lymphocyte ratio (PLR) and the systemic immune-inflammation index (SII), which are thought to better reflect the relative balance between the innate and adaptive immune system (**Figure 2**).

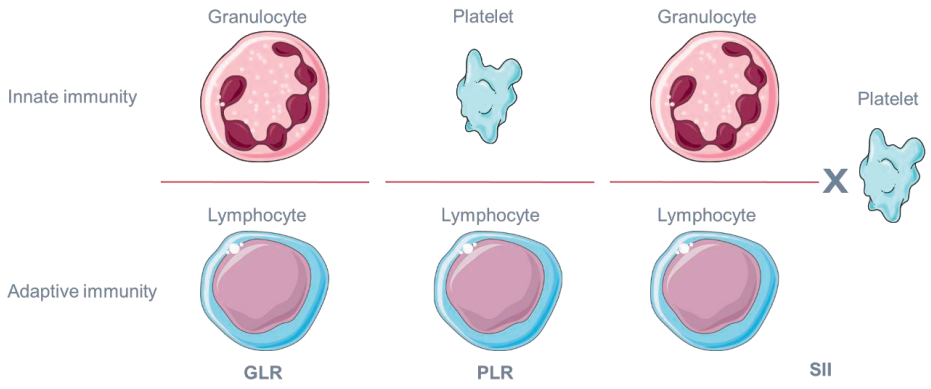

Figure 2. White blood-cell based inflammatory indices reflecting the relative balance between the innate and adaptive immune system: Granulocyte-to-lymphocyte ratio (GLR), the platelet-to-lymphocyte ratio (PLR) and the systemic immune-inflammation index (SII).

To follow-up on the findings of **chapter 2.1**, I performed a two-sample Mendelian randomization study (**chapter 2.2**) examining immune cells and signaling molecules in relation to the risk of AD and hippocampal volume. I further study whether common infectious diseases could be a trigger of low-grade inflammation by studying the relation between *Helicobacter pylori* (**chapter 2.3**) and Herpes Simplex Virus type 1 (**chapter 2.3**) in relation to the risk of dementia.

The immune system is not only a potential determinant for dementia, but also for stroke.[30] Indeed, studies have shown that gene expression changes in the peripheral blood and immune cells of stroke patients can serve as potential diagnostic biomarkers in acute stroke.[31,32] Moreover, randomized clinical trials have also shown potential for anti-inflammatory treatment in reducing cardiovascular risk including stroke.[33,34] **Chapter 3** therefore examines immunity components, the same immune cells and derived ratios as studied in **chapter 2.1**, in relation to stroke. In **chapter 3.1**, I determine the association between immunity components and the risk of atherosclerotic cardiovascular disease, including stroke and coronary heart disease. I furthermore relate these immunity components to arterial calcifications as measures of subclinical atherosclerosis and examine to what extent arterial calcifications serve as a potential mediator in the relation between immunity components and atherosclerotic cardiovascular disease. Finally, in **chapter 3.2** I examine immunity components with carotid atherosclerotic plaque characteristics to study whether immunity components relate to the plaque's vulnerability to rupture.

BRAIN PATHOLOGY

In addition to immunity and inflammation being potentially important factors for AD and stroke, Alois Alzheimer already reported early on plaques and neurofibrillary tangles, after which Kraepelin included AD in his book *Pschiatrie*.[9] To date, amyloid plaques and neurofibrillary tangles are still considered the hallmark features of AD (**Figure 3**).[35] Amyloid plaques contain toxic peptides known as β-*amyloid*, while neurofibrillary tangles contain micro-tubule-associated proteins, known as *tau.*[18]

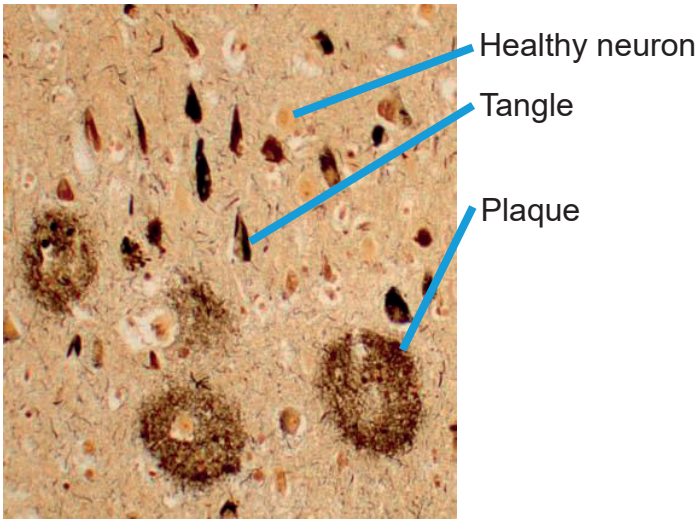

Figure 3. A section of a neocortex from a patient with Alzheimer's disease treated with a silver stain. The tissue shows neuronal cell bodies containing neurofibrillary tangles and neuropil containing amyloid plaques. *Modified from Principles of Neural Science.*

While in AD, extracellular deposits of polymerized β-amyloid peptides create an amyloid plaque, β-amyloid deposition can also occur in the vascular walls, which leads to loss of vessel integrity and can result in cerebral amyloid angiopathy, a condition that increases the risk for stroke caused by bleeding.[36] **Chapter 4.1** of this thesis describes how these biomarkers of AD-related brain pathology as measured in plasma relate to stroke risk in the general population. I furthermore describe the immune system as potential determinant of these markers (**chapter 4.2**) by studying their relation with immune cells. Some of these immune cells, the leukocytes, own a nucleus and thus contain DNA from which we can measure telomere length.[37] As an indicator of oxidative stress and senescence, telomere length is hypothesized to be a biomarker of aging.[38] In **chapter 4.3** I study telomere length as measured from leukocytes as another potential biomarker of AD.

Brain hemodynamics

The brain is highly vulnerable to disturbance of its blood supply.[18] Therefore, blood flow to the central nervous system must efficiently deliver oxygen, glucose, and other nutrients and remove carbon dioxide, lactic acid, and other metabolites.[39] The major vessels that provide the brain of its blood are the carotid arteries and the vertebrobasilar arterial system (**Figure 4**).[18] The brain vasculature has special anatomical and physiological properties that protect the brain from hypoperfusion. Autoregulation involves the alteration of small vessel diameters and is observed in virtually every vascular bed, but it is most pronounced in the brain and kidney.[39] When this protective mechanism fails, ischemia could occur which may lead to neurological dysfunction. When this episode of neurological dysfunction is brief

(<24 hours), we refer to this as a *transient ischemic attack* or TIA. Although most occlusive strokes are caused by atherosclerosis and thrombosis, there are also several other causes, of which subclinical hypoperfusion remains relatively unknown.[40] **Chapter 5** of this thesis is dedicated to studying measures of brain hemodynamics within the general population. First, I describe the association between global brain perfusion and the risk of TIA and ischemic stroke (**chapter 5.1**). In **chapter 5.2**, I then present thyroid function as a potential determinant of brain hemodynamics.

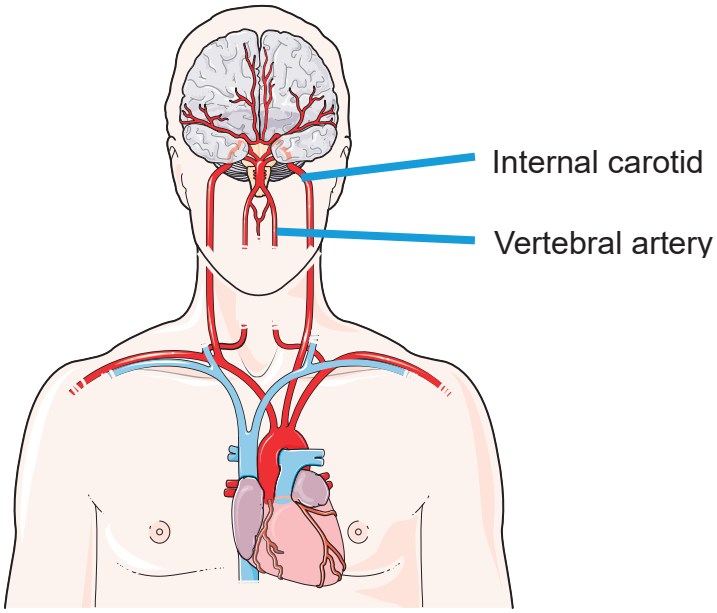

Figure 4. Major arteries supplying the brain of blood. *Modified from smart servier medical art.*

Studying the role of various etiological entities including brain pathology and brain hemodynamics in dementia and stroke provides new opportunities to connect the two diseases. Moreover, I show that targeting immunity and inflammation also point to new directions, and sheds new light on pathological and hemodynamical changes that occur in the brain. In **chapter 6**, the findings described in this thesis are therefore reflected upon from an immunity perspective. I conclude with a discussion of the implications for future research.

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Chapter 2
Immunity and dementia

2.1 Balance between innate versus adaptive immune system

ABSTRACT

Background: Immunity has been suggested to be important in the pathogenesis of dementia. However, the contribution of innate versus adaptive immunity in the development of dementia is not clear. In this study we aimed to investigate 1) the association between components of innate immunity (granulocytes and platelets) and adaptive immunity (lymphocytes) with risk of dementia and 2) the association between their derived ratios (granulocyte-to-lymphocyte ratio [GLR], platelet-to-lymphocyte ratio [PLR], and systemic immune-inflammation index [SII]), reflecting the balance between innate and adaptive immunity, with risk of dementia.

Methods: Blood cell counts were measured repeatedly between 2002-2015 in dementia-free participants of the prospective population-based Rotterdam Study. Participants were followed-up for dementia until 1st January 2016. Joint models were used to determine the association between granulocyte, platelets, and lymphocyte counts, and their derived ratios with risk of dementia.

Results: Of the 8313 participants (mean [standard deviation] age 61.1 [7.4] years, 56.9% women), 664 (8.0%) developed dementia during a median follow-up of 8.6 years. Doubling of granulocyte and platelet counts tended to be associated with an increased risk of dementia (HR [95%CI]: 1.22 [0.89-1.67] and 1.45 [1.07-1.95], respectively). Doubling of the derived ratios GLR, PLR, and SII were all associated with an increased dementia risk (HR [95%CI]: 1.26 [1.03-1.53], 1.27 [1.05-1.53], and 1.15 [0.98-1.34], respectively).

Conclusions: GLR, PLR, and SII are associated with an increased risk of dementia in the general population. This supports the role of an imbalance in the immune system towards innate immunity in the pathogenesis of dementia.

INTRODUCTION

Dementia poses a huge burden on societies in terms of financial costs as well as on individual patients and their caregivers regarding suffering and grief [1]. Dementia is a multifactorial disease, in which various pathologies interact during the long pre-clinical phase, ultimately resulting in its clinical manifestations of cognitive decline and loss of independence. While amyloid depositions, neuronal loss, and vascular damage have long been established as key pathologies underlying dementia[2], recent findings point towards a key role for the immune system [3-5]. The immune system is a highly complex system involving multiple synergistic and antagonistic substrates, yet broadly can be classified into two components, i.e. innate immunity and adaptive immunity [6]. Innate immunity refers to immune responses present at birth, forming a first line of defence, whereas adaptive immunity is acquired during life by exposure to specific antigens [7]. High activity of innate immunity can lead to disrupted neuronal integrity, and ultimately in cell death [8]. Although these components of the immune system work closely together, adaptive immunity is considered to be more neuroprotective than innate immunity, presumably by stimulating phagocytosis of amyloid fibrils [9, 10].

Exact quantification of these opposing components of the immune system is challenging and focus of ongoing research, but recent work from the field of cancer research suggests that easily obtainable laboratory measurements may in fact capture their relative activity levels to a reliable degree [11]. Measuring granulocytes, including the most abundant subtype neutrophils, and platelets provides important markers of the innate immunity, whereas measuring lymphocytes yields information on the adaptive immunity [12, 13]. Furthermore, combining these measurements into ratios, i.e. the neutrophil-to-lymphocyte ratio (NLR), platelet-to-lymphocyte ratio (PLR), and systemic immune-inflammation index (SII), is thought to even better reflect the relative balance between innate and adaptive immunity [11, 14-16]. Previous work on the link between innate versus adaptive immunity and dementia showed higher NLR and PLR in dementia patients compared to healthy individuals [17-19]. Yet, to really understand the role of the immune system in the risk of developing dementia, it is pivotal to study how these markers change during the pre-clinical phase of the disease.

We thus investigated the longitudinal association of markers of the innate versus adaptive immune system with the risk of dementia. The underlying hypothesis was that higher activity of the innate versus adaptive immune system would be associated with an increased risk of dementia. A further methodological novelty of our study was the use of joint modelling that enabled us to study the longitudinal evolution of the various markers during the pre-clinical phase in conjunction with survival analyses.

METHODS

Study population

The present study is embedded in the Rotterdam Study, a prospective population-based cohort study in Rotterdam, the Netherlands. The Rotterdam Study started in 1990 with 7983 persons (response of 78%) aged ≥55 years and residing in the district Ommoord, a suburb of Rotterdam. This first subcohort (RS-I) was extended with a second subcohort (RS-II) in 2000, consisting of 3011 persons (response of 67%) and with a third subcohort (RS-III) in 2006, composed of 3932 persons aged ≥45 years (response of 65%). The design of the Rotterdam Study has been described in detail previously [20]. In brief, participants were examined in detail at study entry and at follow-up visits every three to five years. They were interviewed at home by a trained research nurse, followed by two visits at the research facility for additional interviewing, laboratory assessments, imaging, and physical examinations.

The Rotterdam Study was approved by the Medical Ethics Committee of Erasmus Medical Center and by the board of The Netherlands Ministry of Health, Welfare, and Sports. A written informed consent was obtained from all participants.

Laboratory tests for granulocytes, platelets, and lymphocytes were introduced from 2002 onwards, corresponding with the following assessment rounds in the Rotterdam Study (baseline in this study): i.e. fourth round of RS-I, second round of RS-II, and first round of RS-III, comprising 9994 participants. From these 9994 eligible participants, we excluded those without complete baseline blood measurements (n=1288). Of the remaining participants, we excluded those with a history of dementia (n=52), participants who were insufficiently screened for dementia (n=62), and those without informed consent to assess medical records during follow-up (n=39). Lastly, we excluded participants with missing apolipoprotein E (*APOE*) genotype (n=240), resulting in 8313 participants for analysis (Flowchart in **Figure 1**).

Assessment of blood cell counts and their derived ratios

Fasting blood samples were taken during each visit at the research center with a maximum of three visits during follow-up. Full blood count measurements were performed using the COULTER® Ac·T diff2™ Hematology Analyzer (Beckman Coulter, San Diego, California, USA) directly after blood sample drawn. Laboratory measurements included absolute granulocyte, platelet, and lymphocyte counts in 10^9 per liter. Since neutrophil counts were not available, we used granulocyte count as a reliable proxy given that these are the most abundant subtype of neutrophils [21, 22].

The GLR and PLR were calculated as the ratio of granulocyte count to lymphocyte count, and as the ratio of platelet count to lymphocyte count, respectively. The SII was defined as platelet count times the GLR.

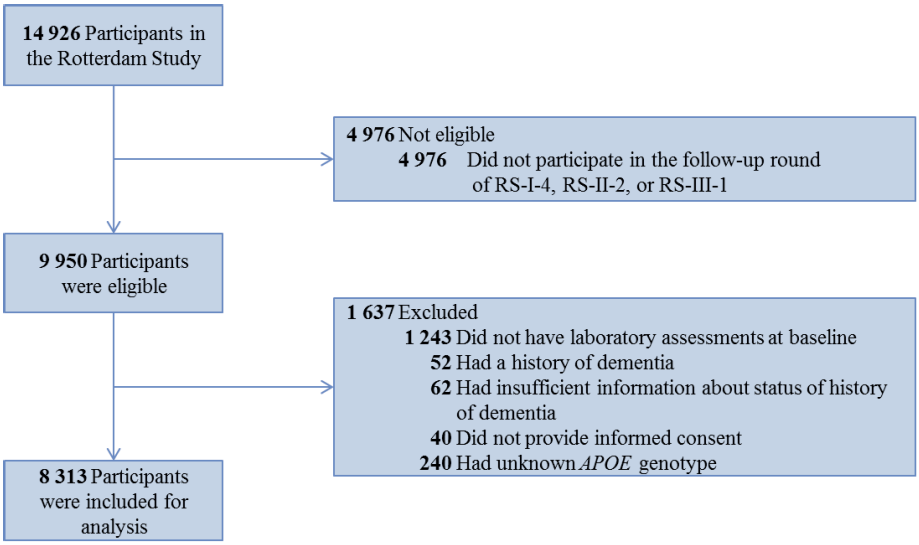

Figure 1 *Flowchart participants for analysis association between blood cell counts and their derived ratios, and dementia.*
Abbreviations: *APOE* = apolipoprotein E.

Assessment of dementia

Participants were screened for dementia at baseline and subsequent center visits with the Mini-Mental State Examination and the Geriatric Mental Schedule organic level [23]. Those with a Mini-Mental State Examination score <26 or Geriatric Mental Schedule score >0 underwent further investigation and informant interview, including the Cambridge Examination for Mental Disorders of the Elderly. The entire cohort was continuously under surveillance for dementia through electronic linkage of the study database with medical records from general practitioners and the regional institute for outpatient mental health care. Available information on clinical neuroimaging was used when required for diagnosis of dementia subtype. A consensus panel led by a consultant neurologist established the final diagnosis according to standard criteria for dementia (Diagnostic and Statistical Manual of Mental Disorders III-revised), Alzheimer's disease (AD, National Institute of Neurological and Communicative Disorders and Stroke and the Alzheimer's Disease and Related Disorders Association), and vascular dementia (National Institute of Neurological Disorders and Stroke and Association Internationale pour la Recherché et l'Enseignement en Neurosciences). Follow-up until 1st January 2016 was virtually complete (93.8% of potential person-years observed).

Other measurements

We assessed education and smoking by interview. Education level was classified into primary education, lower (lower general education, intermediate general education, or lower vocational education), intermediate (intermediate vocational education or higher general education), or higher (higher vocational education or university). Smoking status was categorized as never, former, or current smoker. Body mass index (BMI) was computed from measurements of height and weight (kg/m^2). Diabetes mellitus was defined as use of antidiabetic medication, fasting serum glucose level ≥7.1 mmol/L, or random serum glucose level ≥11.1 mmol/L [24]. History of stroke was assessed by interview and verified by reviewing medical records [25]. *APOE* genotype was determined using polymerase chain reaction on coded DNA samples in RS-I and with a bi-allelic TaqMan assay in the two extensions (RS-II and RS-III) [26, 27]. *APOE-ε4* carrier status was defined as carrier of one or two *APOE-ε4* alleles.

Statistical analysis

We associated the different blood cell counts and their derived ratios with the risk of all-cause dementia using the framework of joint models for longitudinal and survival data. In this way we are able to account for the endogenous nature (i.e. blood cell counts can be measured with error during follow-up and their values at any time point can be affected by an event occurring at an earlier time point) [28] and the correlations in the repeated measurements of granulocyte, platelet, and lymphocyte counts [29].

In order to normalize the skewed distribution of granulocyte, platelet, and lymphocyte counts, and their derived ratios we used a natural logarithmic transformation. Hazard ratios (HRs) with 95% confidence intervals (CIs) were obtained from the joint models using the piecewise-constant baseline hazard, and multiplied with log(2), providing a HR for doubling of the blood cell counts and their ratios. We computed two nested models: Model I was adjusted for baseline age (continuous, centered as age minus mean age) and sex; Model II was additionally adjusted for education, smoking status, BMI (continuous), diabetes mellitus, history of stroke, and *APOE-ε4* carrier status. For assessment of the association between the individual components of the ratios and dementia, we repeated analyses with adjustment for the baseline blood cell counts of the remaining two blood cell types (for instance, the association of granulocyte count with dementia was adjusted for platelet and lymphocyte counts). Follow-up time was used as timescale and started at the first laboratory assessment until date of all-cause dementia diagnosis, death, loss to follow-up, or 1st January 2016, whichever came first. Censoring participants at date of death allowed us to compute cause-specific HRs.

In sensitivity analyses, we repeated all analyses using age as timescale instead of follow-up time to account for potential residual confounding by age and to minimize potential effects of left truncation. We additionally censored for stroke events during follow-up to preclude

that the observed effect may be driven by incident strokes that occurred before dementia diagnosis. Moreover, we investigated the association between the ratios and AD or vascular dementia separately. Lastly, we explored effect modification by stratifying by median age, sex, smoking status, diabetes mellitus, and *APOE-ε4* carrier status.

Multiple imputation was used for missing covariates (maximum of 0.99%), with five imputed datasets based on other covariates and the outcome. Rubin's method was used for pooled HRs and 95% CIs [30]. Two-sided P <.05 was considered statistically significant. Statistical analyses were performed using the R packages 'survival', 'nlme', 'JM', and 'JMbayes' in RStudio Version 3.3.2 [28, 29, 31, 32].

RESULTS

Characteristics of included and excluded study participants are presented in **Table 1**. An overview of the median blood cell counts and blood cell-based ratios per assessment round is shown in Supplementary Table 1. Mean age of included study participants was 61.1 years and 56.9% were women. During a median follow-up of 8.6 years (70273 person-years), 664 participants developed all-cause dementia (543 AD, 31 vascular dementia) with an incidence rate of 9.4 (95% CI, 8.7-10.2) per 1000 person-years.

Higher levels of granulocytes reflecting higher innate immunity were associated with an increased risk of dementia, but only after correcting for the platelet and lymphocyte counts (HR for doubling granulocyte count [95% CI] = 1.33 [0.99-1.79], **Table 2**). Doubling of platelets was associated with an increased risk of dementia (HR [95% CI] = 1.48 [1.11-1.96]). Regarding adaptive immunity, higher levels of lymphocytes were associated with a decreased risk of dementia (HR for doubling lymphocyte count [95% CI] = 0.80 [0.64-0.99]).

Higher levels of GLR, PLR, and SII were associated with an increased dementia risk (HR [95% CI] for doubling GLR = 1.34 [1.10-1.63]; for PLR = 1.29 [1.08-1.55]; for SII = 1.18 [1.02-1.39], respectively [**Table 2**]). Risk estimates were comparable when using the adjusted model and when using age as timescale instead of follow-up time.

Censoring for stroke did not meaningfully change the risk estimates (**Table 3**). Higher levels of platelets showed a slightly stronger association with AD compared with all-cause dementia, whilst the association with granulocytes was less pronounced for AD. Risk estimates for all-cause dementia and AD were comparable for the ratios. For vascular dementia, risk estimates regarding the individual blood cell components and their derived ratios were more pronounced than for all-cause dementia, but small numbers led to wider confidence intervals (n=31).

Table 1 Baseline characteristics of the included and excluded study participants.

Characteristic	Included participants (N = 8313)	Excluded participants (N = 1528)#	
		No blood measurements (N = 1288)	Unknown APOE genotype (N = 240)
Age, year, mean (SD)	61.1 (7.4)	72.6 (11.8)	61.7 (8.2)
Women	4729 (56.9)	845 (65.6)	160 (66.7)
Education			
Primary	908 (11.0)	233 (18.4)	25 (11.7)
Lower	3329 (40.3)	537 (42.4)	95 (44.4)
Intermediate	2429 (29.4)	336 (26.5)	57 (26.7)
Higher	1588 (19.2)	161 (12.7)	37 (17.3)
Body mass index, kg/m^2, mean (SD)	27.6 (4.3)	27.6 (4.5)	28.2 (4.8)
Smoking status			
Current	1595 (19.3)	308 (24.4)	57 (24.5)
Former	4191 (50.7)	550 (43.6)	106 (45.5)
Diabetes mellitus	501 (6.0)	136 (10.7)	15 (6.3)
History of stroke	305 (3.7)	54 (4.2)	11 (4.6)
APOE-ε4 carrier status	2328 (28.0)	244 (30.8)	
Blood cell types, 10^9/L, median (IQR)			
Granulocytes	3.8 (1.6)		4.0 (1.7)
Platelets	263 (84)		277 (87)
Lymphocytes	2.2 (0.8)		2.3 (0.9)
Blood cell-based ratios, median (IQR)			
Granulocyte-to-lymphocyte ratio	1.7 (0.9)		1.7 (0.8)
Platelet-to-lymphocyte ratio	120 (55)		119 (54)
Systemic immune-inflammation index	455 (280)		473 (312)

Abbreviations: *APOE*, apolipoprotein E; IQR, interquartile ratio; N = number of participants; SD, standard deviation.

Values are shown before multiple imputation and therefore not always add up to 100%.

Data are presented as number (percentage) of participants unless otherwise indicated.

Excluded participants in this table only include those participants who were excluded due no complete blood measurements or unknown *APOE-ε4* carrier status.

Table 2 Association between blood cell counts and derived ratios, and risk of all-cause dementia.

Laboratory assessment#	All-cause dementia (n/N = 664/8313)	
	Model I	**Model II**
	HR (95% CI)	**HR** (95% CI)
Granulocytes	1.14 (0.87-1.50)	1.07 (0.80-1.43)
Corrected for platelets and lymphocytes	1.33 (0.99-1.79)	1.22 (0.89-1.67)
Platelets	1.48 (1.11-1.96)*	1.43 (1.08-1.90)*
Corrected for granulocytes and lymphocytes	1.48 (1.10-2.00)*	1.45 (1.07-1.95)*
Lymphocytes	0.80 (0.64-0.99)*	0.81 (0.64-1.03)
Corrected for granulocytes and platelets	0.76 (0.61-0.96)*	0.78 (0.62-1.00)
Granulocyte-to-lymphocyte ratio	1.34 (1.10-1.63)*	1.26 (1.03-1.53)*
Platelet-to-lymphocyte ratio	1.29 (1.08-1.55)*	1.27 (1.05-1.53)*
Systemic immune-inflammation index	1.18 (1.02-1.39)*	1.15 (0.98-1.34)

Abbreviations: CI = confidence interval; HR = hazard ratio; n = number of incident dementia events; N = number of participants for analysis. Model I is adjusted for age and sex. Model II is adjusted for age, sex, education, smoking status, body mass index, diabetes mellitus, history of stroke, and *APOE4* ε4 carrier status.

[#] All types of blood cells and their derived ratios were natural logarithmic transformed.

[*] Indicates statistically significant result.

Stratified analyses showed that the association between the ratios and dementia was particularly pronounced in participants aged below the median age of 65.4 years, women, and non-smokers (**Figure 2**). However, formal interaction terms did not reach statistical significance. Also, no significant effect modification was observed across different strata of these variables for the association between granulocyte, platelet, and lymphocyte counts, and risk of dementia (**Figure 3**).

Table 3 Association between blood cell counts derived ratios, and risk of all-cause dementia and dementia subtypes.

Laboratory assessment#	All-cause dementia, censored for stroke (n/N = 579/8008)	Alzheimer's disease (n/N = 543/8313)	Vascular dementia (n/N = 31/8313)
	HR (95% CI)	HR (95% CI)	HR (95% CI)
Granulocytes	1.13 (0.83-1.56)	1.03 (0.75-1.42)	1.99 (0.52-7.55)
Corrected for platelets and lymphocytes	1.36 (0.96-1.93)	1.12 (0.79-1.58)	1.92 (0.44-8.41)
Platelets	1.45 (1.07-1.96)*	1.59 (1.17-2.17)*	3.86 (1.02-14.6)*
Corrected for granulocytes and lymphocytes	1.47 (1.07-2.02)*	1.63 (1.18-2.27)*	3.39 (0.84-13.7)
Lymphocytes	0.80 (0.62-1.02)	0.85 (0.66-1.10)	0.76 (0.25-2.30)
Corrected for granulocytes and platelets	0.76 (0.58-0.98)*	0.81 (0.62-1.06)	0.64 (0.20-2.03)
Granulocyte-to-lymphocyte ratio	1.33 (1.07-1.65)*	1.17 (0.95-1.46)	1.85 (0.74-4.62)
Platelet-to-lymphocyte ratio	1.31 (1.07-1.60)*	1.30 (1.06-1.60)*	1.99 (0.82-4.81)
Systemic immune-inflammation index	1.19 (1.01-1.41)*	1.15 (0.97-1.37)	1.77 (0.87-3.63)

Abbreviations: CI = confidence interval; HR = hazard ratio; n = number of incident dementia events; N = number of participants for analysis. Models are adjusted for age, sex, education, smoking status, body mass index, diabetes mellitus, history of stroke, and *APOE4* ε4 carrier status.

[#] All types of blood cells and their derived ratios were natural logarithmic transformed.

[†] Number of participants for analysis is 8313 minus participants with a history of stroke (n=305).

[*] Indicates statistically significant result.

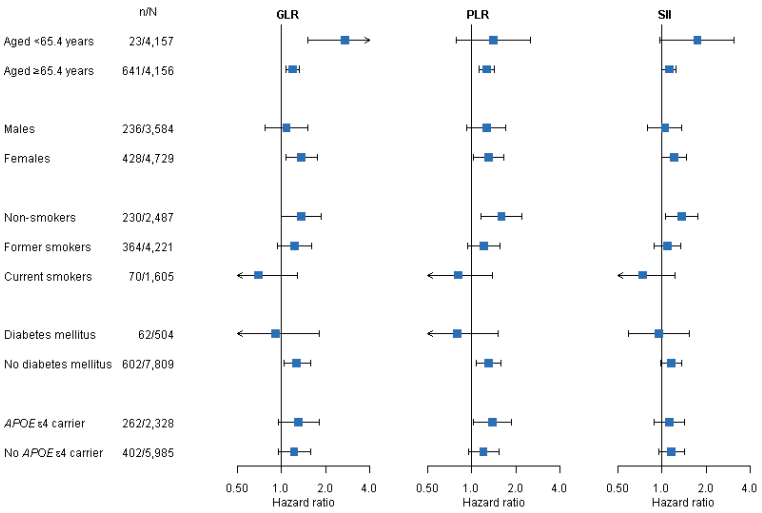

Figure 2 *Forest plots of the association of the GLR, PLR, and SII, and risk of dementia.* Hazard ratios are shown in logarithmic scale with stratification by median age, sex, smoking status, diabetes mellitus, and *APOE-ε4* carrier status.
Abbreviations: APOE, apolipoprotein E; n = number of incident dementia events; N = number of participants for analysis.

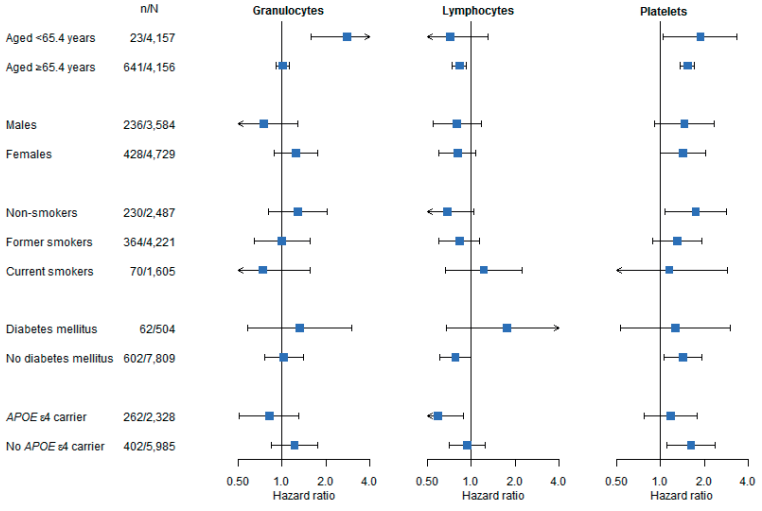

Figure 3 *Forest plots of the association of granulocytes, platelets, and lymphocytes, and the risk of dementia.* Hazard ratios are shown in logarithmic scale with stratification by median age, sex, smoking status, diabetes mellitus, and *APOE-ε4* carrier status. Abbreviations: APOE, apolipoprotein E; n = number of incident dementia events; N = number of participants for analysis.

DISCUSSION

In this population-based study, we found that higher levels of granulocyte and platelet counts are related to an increased risk of dementia, whereas a higher lymphocyte count is associated with a decreased dementia risk. Furthermore, higher levels of their derived ratios, i.e. GLR, PLR, and SII are associated with an increased risk of all-cause dementia, including its subtype AD and even more with vascular dementia.

Activation of the immune systems can result in inflammation by production of different cytokines [33]. These cytokines can act as a link between the innate and the adaptive immune system, having pro- or anti-inflammatory effects depending on the type of cytokine [34]. A recent meta-analysis of 175 studies suggests that AD is accompanied by an inflammatory response and that this can be reflected by a variety of systemic cytokines, for instance interferon-γ, interleukin(IL)-2, and in particular IL-6, of which dysregulation has been associated with multiple chronic inflammatory diseases [35, 36]. It is now recognized that systemic inflammation can trigger or exacerbate the inflammatory environment of the brain, thereby contributing to chronic neuroinflammation and neurodegeneration [37]. A plausible explanation for the occurrence of this chronic neuroinflammation in (pre)demented individuals involves a disruption of a process called resolution [38]. Resolution is an active process that halts the acute phase of inflammation and restores tissue homeostasis. The acute inflammatory phase is usually initiated in response to infection, neoplasia, tissue injury, or other major homeostatic stressors. This phase is accompanied by the increased release of pro-inflammatory mediators such as prostaglandins, leading to leukocyte recruitment. Normally, resolution would clear the recruited granulocytes [39]. However, it has been shown that failure of resolution, induced by any chronic inflammatory state, is associated with an over-active innate immune system, resulting in the development of chronic inflammation, which could subsequently lead to AD [38, 40, 41]. Our finding that an increase in the granulocyte count, resulting in a higher GLR and SII, is associated with an increased risk of dementia could therefore support the role of insufficient resolution in the pathogenesis of dementia.

Only few studies examined the interplay between the innate and adaptive immunity by studying levels of these blood cell-based ratios in dementia patients. Two cross-sectional studies showed that NLR and PLR were elevated in AD patients compared to dementia-free controls [17, 18]. In contrast, a longitudinal study assessing the trajectory of NLR found no significant difference in its longitudinal evolution between AD patients and dementia-free participants [19]. Although they examined differences between AD patients and dementia-free controls, they did not investigate the risk of developing dementia in dementia-free participants in relation to their levels of NLR. In the present study, we did take the time until dementia into account by a joint modelling approach and were therefore able to assess the risk of dementia in relation to the change of blood cell counts and their derived ratios.

Interestingly, recent evidence shows that the NLR and PLR are partly genetically determined with 36% estimated heritability for NLR and 64% for PLR in a healthy population [42]. Moreover, different single nucleotide polymorphisms (SNPs) identified through genome-wide association study (GWAS) were significantly related to the PLR phenotype, but not to NLR [43]. Importantly, some but not all of these SNPs were also related to platelet, indicating that these SNPs capture the interplay between platelets and lymphocytes. Thus far no GWAS for SII has been performed. Exploring the dementia risk by genetically predicted blood cell-based ratios may provide more insight in the causal role of immunity in dementia.

Strengths of our study include the population-based setting and the thorough follow-up for dementia. Another strength is the prospective design of this study, with the blood cell counts being measured at multiple time points. Using an innovative statistical method, we combined these repeated measurements with dementia as survival outcome. Moreover, we used blood cell counts and their derived ratios, which low-cost and easy to implement in the clinic and other research settings. Although these ratios are proven to be associated with chronic systemic inflammation, we need to emphasize that it is unknown whether higher levels of GLR, PLR, and SII are functional and cause higher levels of pro-inflammatory cytokines. To identify the actual involved immune cell populations determination of different cytokines is still needed. Furthermore, the innate and adaptive immune systems are overlapping, making it difficult to completely distinguish their separate effects. In addition, we used the granulocyte count as proxy for the neutrophil count. Although the relative proportion of neutrophils compared to eosinophils and basophils may be lower in persons with several specific diseases such as parasitic infections, asthma, or immune diseases, neutrophils are generally the most important subtype of granulocytes. If anything, misclassification of the granulocytes would be non-differential and would therefore lead to underestimation of the estimates [11]. In addition, we cannot rule out reversed causality, i.e. that dementia is subclinical at time of the laboratory assessments and causes higher levels of GLR, PLR, and SII. Lastly, we did not have the power to study other neurodegenerative diseases beyond dementia such as Parkinson's disease or amyotrophic lateral sclerosis. It would be interesting for future studies to also investigate the relation between inflammation and these diseases.

In conclusion, higher levels of the ratios GLR, PLR, and SII are associated with an increased risk of developing dementia in the general population. Higher activation of the innate immune system reflected by higher levels of granulocytes and platelets, is associated with an increased dementia risk, while the adaptive immune system is suggested to be more neuroprotective. These findings support the role of dysregulation of the immune systems in the pathogenesis of dementia. Further studies are warranted to assess during which phase of the pathogenesis of dementia immunity is involved and to assess causality in order to develop prevention and therapeutic strategies.

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SUPPORTING INFORMATION

Supplementary Table 1 Overview of median blood cell counts and blood cell-based ratios measured per Rotterdam Study assessment round.

Laboratory assessment	First assessment round# (N=8313)	Second assessment round† (N=5663)	Third assessment round* (N=1886)
Blood cell types, 10^9/L, median (IQR)			
Granulocytes	3.8 (1.6)	4.0 (1.6)	3.6 (1.5)
Platelets	263 (84)	262 (83)	224 (75)
Lymphocytes	2.2 (0.8)	2.2 (0.8)	1.9 (0.9)
Blood cell-based ratios, median (IQR)			
Granulocyte-to-lymphocyte ratio	1.7 (0.9)	1.8 (0.9)	1.9 (1.1)
Platelet-to-lymphocyte ratio	120 (55)	116 (53)	117 (59.1)
Systemic immune-inflammation index	455 (280)	461 (283)	421 (290)

Abbreviations: IQR, interquartile ratio; N = number of participants; RS = Rotterdam Study.

[#] The first measurement corresponds with the fourth round of RS-I, second round of RS-II, and first round of RS-III.

[†] The second measurement corresponds with the fifth round of RS-I, third round of RS-II, and second round of RS-III.

[*] The third measurement corresponds with the sixth round of RS-I and the fourth round of RS-II.

2.3 *Helicobacter pylori*

ABSTRACT

Background: *Helicobacter pylori* infection might increase risk of dementia, but available evidence is inconsistent and longitudinal studies are sparse. We investigated the association between *H. pylori* serology and dementia risk in a population-based cohort.

Methods: Between 1997-2002, we measured *H. pylori* serum IgG titers in 4215 non-demented participants of the Rotterdam Study with a mean age of 69 years. We determined the association between *H. pylori* at baseline and dementia incidence until 2015, per natural log(U/mL) increase in titer, and for seropositive/seronegative, using Cox models adjusting for cohort, sex, age, education and cardiovascular risk factors.

Results: During a median follow-up of 13.3 years, 529 participants developed dementia of whom 463 had Alzheimer's disease (AD). *H. pylori* was not associated with risk of dementia (hazard ratio [95% confidence interval] for antibody titer: 1.04 [0.90-1.21]; for seropositivity 1.03 [0.86-1.22]), nor AD.

Discussion: In this community-dwelling population, *H. pylori* was not associated with dementia risk.

INTRODUCTION

Helicobacter pylori (*H. pylori*) is a gram-negative bacillus that colonizes the stomach and is estimated to infect half of the world's population [1]. The bacterium is generally acquired during childhood by oral ingestion. In adults, the infection is usually chronic and will not heal without specific therapy. The clinical course, however, is highly variable. While in some individuals infections remain asymptomatic, others may develop serious gastric complications, such as ulcers or gastric carcinoma [2].

In addition, recent evidence suggests that *H. pylori* infection might be associated with extra-gastric diseases including dementia [3-5]. This may be due to detrimental consequences of *H. pylori* associated anemia, inflammation and hyperhomocysteinemia on vascular and neuronal health [6, 7]. A recent systematic review and meta-analysis [8] showed a 71% increased risk of dementia with *H. pylori* infection, but heterogeneity across studies was high. This may relate to differences in (geographical) setting and study design, with current evidence mainly arising from cross-sectional studies. We aimed to investigate the association of *H. pylori* with dementia in a prospective population-based cohort study.

METHODS

Design and study population

This study was embedded within the Rotterdam Study, a prospective population-based cohort study among middle-aged and elderly individuals in the Netherlands [9]. A detailed description is provided in the online supplement. Established in 1990, participants were invited every 4-5 years to undergo follow-up examinations at the research center. Between 1997 and 2002, a total of 7444 participants from two subcohorts visited the research center, of whom 4215 dementia-free subjects provided blood samples for measurement of *H. pylori* titer (Figure S1).

The Rotterdam Study has been approved by the medical ethics committee according to the Population Study Act Rotterdam Study and written informed consent was obtained.

Assessment of anti-H. pylori antibodies

Blood was drawn at baseline. To obtain serum and plasma, tubes were centrifuged according to a protocol standardising time and conditions from the drawing of blood to centrifugation. All samples were snap frozen at –196°C using liquid nitrogen and stored at –80°C. Anti–*H. pylori* serum IgG antibody titers were measured in 2011 using commercial enzyme immunoassays (Pyloriset EIA-G III ELISA; Orion) as described earlier [10]. We used anti-*H. pylori* serum IgG antibody titers primarily as a continuous variable. In addition, seropositivity was

defined as an anti–*H. pylori* IgG titer equal to or greater than 20 U/mL, according to the manufacturer's recommendation.

Ascertainment of incident dementia

Participants were screened for dementia at baseline and subsequent center visits with the Mini-Mental State Examination and the Geriatric Mental Schedule organic level. Those with a Mini-Mental State Examination score <26 or Geriatric Mental Schedule score >0 underwent further investigation and informant interview, including the Cambridge Examination for Mental Disorders of the Elderly. In addition, the entire cohort was continuously under surveillance for dementia through electronic linkage of the study center with medical records from general practitioners and the regional institute for outpatient mental health care. A consensus panel headed by a consultant neurologist established the final diagnosis according to standard criteria for dementia (DSM-III-R) and AD (National Institute of Neurological and Communicative Disorders and Stroke– Alzheimer's Disease and Related Disorders Association). Follow-up until 1st January 2015, was virtually complete (99.1% of potential person-years in the original cohort and for 97.0% of potential person-years in the extended cohort). Within this period, participants were censored at date of dementia diagnosis, death, loss to follow-up, or administrative censoring date, whichever came first.

Covariates

Potential confounding factors for dementia were chosen on the basis of previous literature [8, 11]. In all models, we adjusted for cohort, sex and age at baseline. In multivariate adjusted models, we additionally adjusted for education, smoking, systolic and diastolic blood pressure, anti-hypertensive drug use, body mass index (BMI), cholesterol, high-density lipoprotein (HDL) cholesterol, triglycerides, APOEε carrier status, stroke, diabetes mellitus, ethnicity and serum lipid reducing agents at baseline. APOEε carrier status was corrected for since a study has shown that *H. pylori* and ApoE 4 polymorphism may be associated with dysphagic symptoms in older adults [12]. Assessment methods of the covariates are described in the supplemental materials. Blood samples for determination of levels of hemoglobin, homocysteine, CRP, interleukin-6, α1-antitrypsin (α1-AT), lipoprotein-associated phospholipase A2 activity and fibrinogen were obtained at the research center.

Statistical analysis

Because of a skewed distribution of anti-*H. pylori* serum IgG antibody titers, we performed a natural log transformation. Differences in baseline characteristics between the *H. pylori* positive and negative groups were assessed using Student's *t*-test and chi-squared test. We determined the association between *H. pylori* (continuously as well as dichotomized for seroprevalence) and risk of dementia, using Cox regression models, adjusting for age, sex,

study cohort, education and cardiovascular risk factors. The proportional hazards assumption was met.

We repeated analysis for Alzheimer's disease only and used higher cut-offs for seroprevalence (in steps of 5 U/mL from the manufacturer's recommended cut-off) to explore differential effects among individuals with more profound antibody response. We assessed interaction by age, sex or medication use by stratification and testing for multiplicative interaction. In addition, we tested for multiplicative interaction between *H. pylori* titer and CRP to assess if there is a difference in dementia risk between more severe infected and less severely infected groups. Finally, we computed Pearson correlation coefficients for the association of *H. pylori* titer with levels of hemoglobin, homocysteine and CRP, with homocysteine and CRP both natural log transformed. In addition to CRP, which was available for all participants, we also determined the correlation between *H. pylori* and four other inflammatory biomarkers that have previously been related to dementia risk [13], and were available in a subsample of approximately 500 participants with *H. pylori* measurement.

All analyses were performed using IBM SPSS Statistics version 21.0 (IBM Corp., Armonk, NY).

RESULTS

Baseline characteristics of the study population are presented in Table 1. During a mean follow-up of 10.6 (±4.5) years and median follow-up of 13.3 years (interquartile range of 5.9) with 47,664 person-years, 529 individuals were diagnosed with dementia of whom 463 had AD.

Serum antibody titer of *H. pylori* was not associated with risk of dementia (hazard ratio (HR), 95% confidence interval (CI), per log unit increase: 1.04, 0.90-1.21). Similarly, *H. pylori* seroprevalence was not associated with dementia risk (HR 1.03, 0.86-1.22). These results were similar for AD (Table 2) and robust for different antibody titer cut-offs defining seroprevalence (Online supplement Figure S2). Risk estimates tended to be higher in older participants and in men, albeit interaction terms were not statistically significant (p = 0.383 and p = 0.142, respectively; Figure S3). We observed no significant interaction between *H. pylori* serum IgG titer or use of antibiotics and anti-acids on dementia risk (p = 0.449) (Figure S3), nor with CRP (p = 0.974). Antibody titers of *H. pylori* showed only very weak correlation with concurrent levels of hemoglobin, homocysteine and CRP (r = 0.004 and p = 0.835, r = 0.086 and p < 0.01 and r = 0.053 and p = 0.001, respectively). Finally, interleukin-6 (IL6), α1-AT, lipoprotein-associated phospholipase A2 activity and fibrinogen showed no meaningful correlations with *H pylori* (r = 0.039 for IL6; r = 0.080 for α1-AT; r = -0.067 for lipoprotein-associated phospholipase A2 and r = 0.004 for fibrinogen; all P >0.05).

Table 1. Baseline characteristics of the Rotterdam Study cohort

	Study population for analysis (N = 4215)	H. pylori positive (N = 2088)	H. pylori negative (N = 2127)	P-value for difference
Age, years	68.4 (8.6)	66.4 (7.5)	65.1 (7.4)	<0.001
Female	2288 (54.3)	1093 (52.3)	1195 (56.2)	0.012
Caucasian ethnicity	4140 (98.2)	2056 (98.5)	2081 (97.8)	0.161
Education				<0.001
Primary education	537 (12.7)	325 (15.6)	215 (10.1)	
Lower/intermediate general education	1873 (44.4)	944 (45.2)	928 (43.6)	
Intermediate vocational education	1244 (29.5)	602 (28.8)	642 (30.2)	
Higher vocational education	561 (13.3)	217 (10.4)	342 (16.1)	
Smoking				0.006
Never smoker	1335 (31.7)	615 (29.5)	719 (33.8)	
Former, now non-smoker	2130 (50.5)	1077 (51.6)	1051 (49.4)	
Current smoker	759 (17.8)	396 (19.0)	357 (16.8)	
Systolic blood pressure, mm Hg	143.4 (21.2)	143.8 (21.2)	143.1 (21.2)	0.284
Diastolic blood pressure, mm Hg	76.9 (11.2)	76.6 (11.3)	77.1 (11.1)	0.117
Anti-hypertensive medication	1488 (35.3)	757 (36.3)	728 (34.2)	0.178
Body mass index, kg/m^2	27.0 (3.9)	27.0 (3.8)	27.0 (4.0)	0.495
Diabetes mellitus	579 (13.7)	276 (13.2)	304 (14.3)	0.333
Cholesterol, mmol/L	5.8 (1.0)	5.8 (1.0)	5.8 (1.0)	0.939
High-density lipoprotein, mmol/L	1.4 (0.4)	1.3 (0.4)	1.4 (0.4)	<0.001
Triglycerides, mmol/L	1.6 (0.8)	1.6 (0.8)	1.6 (0.8)	0.537
Lipid lowering medication	572 (13.6)	291 (13.9)	288 (13.5)	0.742
History of stroke	163 (3.9)	85 (4.1)	78 (3.7)	0.549
Apolipoprotein E genotype				0.701
ε3 homozygote	2478 (58.8)	1218 (58.3)	1264 (59.4)	
ε2/ε2 or ε2/ε3	587 (13.9)	299 (14.3)	288 (13.5)	
ε4/ε4, ε3/ε4 or ε2/ε4	1149 (27.3)	571 (27.3)	575 (27.0)	
H. pylori serum IgG antibody titers, U/mL	19.6 (11.5; 92.2)	94.0 (36.0; 238.9)	11.5 (10.2; 14.2)	<0.001
H. pylori positive seroprevalence	2088 (49.5)			

Abbreviations: N = number of subjects; *H. pylori* = Helicobacter pylori.

Data presented as mean (standard deviation) for continuous variables and number (percentages) for categorical variables. *H. pylori* serum IgG antibody titers is presented as median (interquartile range). Differences in baseline characteristics between the *H. pylori* positive and negative groups were assessed using Student's *t*-test and chi-squared test.

Table 2. *H. pylori* infection and the risk of dementia: longitudinal results

	All dementia n/N = 529/4215 HR, 95% CI	Alzheimer n/N = 463/4215 HR, 95% CI
Model I		
H. pylori serum IgG, per log(U/mL)* **increase**	1.07, 0.92-1.23	1.11, 0.94-1.30
H. pylori seroprevalence, positive versus negative	1.03, 0.87-1.23	1.08, 0.89-1.31
Model II		
H. pylori serum IgG, per log(U/mL)* **increase**	1.04, 0.90-1.21	1.06, 0.90-1.25
H. pylori seroprevalence, positive versus negative	1.03, 0.86-1.22	1.06, 0.87-1.29

Abbreviations: n = number of cases; N = number of persons at risk; HR = hazard ratio; CI = confidence interval; *H. pylori* = Helicobacter pylori.

Cox regression model I: Adjusted for age, sex and study cohort.

Cox regression model II: Adjusted for age, sex, study cohort, education, smoking, systolic and diastolic blood pressure, anti-hypertensive drug use, body mass index (BMI), cholesterol, high-density lipoprotein (HDL) cholesterol, triglycerides, APOEε carrier status, stroke, diabetes mellitus, ethnicity and serum lipid reducing agents.

*Natural log transformed because of right skewed distribution.

DISCUSSION

In this prospective cohort study, we found no association between *H. pylori* infection and risk of all-cause dementia or AD.

There are only two other longitudinal studies performed on this association, which show a hazard ratio of 1.46 (CI 1.01-2.11) [14] and 1.51 (CI 1.25-1.82) [15] for developing dementia. These findings are in contrast with our results, but several potential explanations, including methodological differences, may clarify this contrast. For instance, the community-based PAQUID cohort study performed in France [14] had an older study population (65 years and older) compared with our cohort of 55 years and over. Since we show a tendency for an increased risk in older age groups (Figure S3), an older cohort may indeed show an effect of *H. pylori* on dementia. Also, the PAQUID cohort selected their study sample on being followed-up for 20 years, thereby selecting the survivors, which is more susceptible to selection bias. The other longitudinal study is a Taiwanese registry study [15] using medical information from a nationwide population-based dataset. The study population was aged ≥40 years and followed during 1998–2010. In this retrospective cohort study, dementia diagnosis was therefore based on registries instead of multiple-stage designs with in-person screening for dementia diagnosis, making dementia diagnosis less reliable. In addition, results are prone to confounding due to the inherent shortcomings of the NHRI database. Also, participants with newly diagnosed *H. pylori* were identified with ICD-9 codes, potentially including the symptomatic *H. pylori* cases which may be

more virulent and thereby observing a stronger effect of *H. pylori* on dementia risk in the overall population.There are also potential biological explanations for the differences across studies, such as geographical differences. Studies have found that the rate of colonization with cagA(+) *H. pylori* strains has become very low in the Netherlands [16, 17]. CagA is a highly antigenic protein that is associated with a prominent inflammatory response [18]. This finding suggests that the *H. pylori* infections in the Netherlands may have low virulence in general compared with other countries, leading to less complications. For example, in studies comparing dyspeptic patients, studies indeed report higher prevalence rates of cagA+ strains in France (88%) [19] and Taiwan (99%) [20] compared to 78% in The Netherlands in 1996 [21] with a Dutch study from 2013 showing a sharp age specific decline in CagA+ from 38% to 14% in randomly selected blood donors [17]. This might be reflected in the higher risk estimates in older participants in our study, who would have been exposed more to these highly virulent strains in earlier years, suggesting a cohort effect. Thus, infection-induced cognitive decline can be supported by this finding.

Mechanistically wise, there are several hypotheses regarding the *H. pylori* and dementia association. Prior studies have suggested altered tau phosphorylation [18], vitamin B deficiency (and consequent hyperhomocysteinemia) [7, 22], systemic inflammation [23, 24], and anemia [25] as potential mechanisms by which *H. pylori* could increase risk of dementia. Although no studies have directly assessed these factors as potential intermediates, the lack of any correlation between *H. pylori* and homocysteine, inflammatory markers (CRP, IL6, α1-AT, lipoprotein-associated phospholipase A2 activity, fibrinogen) and hemoglobin levels in our study might also support the notion of a less virulent *H. pylori* strain that is currently prevalent in The Netherlands. This in turn could explain why we did not observe an association of *H. pylori* with risk of dementia.

The role of infectious diseases in the development of dementia has been longer known with diseases such as neurosyphilis and acquired immunodeficiency syndrome (AIDS) being accepted infectious causes [26].However, also more frequently occurring diseases such as herpes virus, toxoplasmosis and cryptococcus have been shown to have a relationship with dementia [27]. It is postulated that since systemic infection can provoke the enhanced synthesis of inflammatory mediators in the brain, infectious diseases may promote the onset of dementia [28]. Thus, even more common infections which an individual is unable to resolve adequately could lead to chronic inflammation and subsequently neuroinflammation [29]. It is therefore important for future studies to identify such sources of inflammation and eventually identify individuals with increased susceptibility for infections for treatment and prevention purposes.

Several limitations of this study should be taken into account. First, the diagnostic test for detecting *H. pylori* infection has the disadvantage that antibodies are present in blood even after eradication of *H. pylori*. Therefore, current and past infection cannot be distinguished, so we cannot rule out the presence of information bias in the form of misclassification of

the exposure. However we do not expect that the association with incident dementia would be affected, since the timing of infection may only be shifted for several months or years. An alternative for future studies would be the stool antigen test, which proves to be more accurate and still non-invasive compared to the serology test [30]. Second, considering the differences in virulence factors of the bacterium and treatment strategies in different countries, our findings may not be generalizable to other geographical regions. To take differences in virulence factors into account, future studies could assess CagA phenotypes in *H. pylori.* Third, the invasiveness of lumbar puncture prevents cerebrospinal fluid (CSF) sampling in participants in the Rotterdam Study and therefore we have not been able to measure *H. pylori* IgG antibodies in CSF. Although CSF measurements are likely more reflective of pathology within the CNS, studies show a larger mean concentration of anti-*H. pylori* IgG concentrations both in CSF as well as in serum in AD patients compared to controls [31, 32]. This finding supports the systemic effect of the infection, which can be thus measured in serum. Finally, the length of time with the infection could not be accounted for in this present study, which may also contribute to the age-related trend for infection-induced cognitive decline that is observed.

In conclusion, we have found no evidence of an association between *H. pylori* infection and risk of all-cause dementia or AD in our Dutch population. This could be due to low virulence of *H. pylori* in the Netherlands. Future studies are warranted to further elucidate the role of common infectious diseases in the pathophysiology of Alzheimer's disease and thereby identifying novel targets for prevention and treatment.

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SUPPORTING INFORMATION

Methods

Study population

Of the 10,215 invited inhabitants, 7,984 agreed to participate in the baseline examinations. In 2000, the cohort was extended with 4,472 invitees of whom 3,011 agreed to participate. From the original cohort, we used the third examination cycle as baseline due to availability of *H. pylori* and homocysteine data. This cohort contained 1892 participants that were included in the analyses based on dementia and *H. pylori* data availability. In the extended cohort, 2408 participants were included in the analyses (Figure S1).

Covariates

All covariates were measured at baseline. Since the study population included 2 cohorts from the Rotterdam Study, cohort was used as a covariate. Educational level was categorized as low (primary education or lower vocational education), intermediate (secondary education or intermediate vocational education), and high educational level (higher vocational education or university). Smoking habits were categorized as current, former and never smoking. Blood pressure was measured twice at the right arm in sitting position at the research center and the average of 2 blood pressure readings was used. BMI was calculated as weight in kilograms per height in meters squared. Total cholesterol and HDL cholesterol were measured in serum in mmol/l. For the anti-hypertensive drugs and serum lipid reducing agents, participants presented all the medication they used in the past week during the home interview. Trained research assistants registered drug names, dose, and indication on a structured data entry form. APOE genotype was determined using polymerase chain reaction on coded DNA samples.

APOEε carrier status was defined as low risk for dementia (2 ε3 alleles), intermediate risk for dementia (2 ε2 alleles or 1 ε3 allele and 1 ε2 allele) and high risk for dementia (1 or 2 ε4 alleles). Diabetes mellitus was defined as a fasting serum glucose level ≥7.0 mmol/L, non-fasting serum glucose level ≥11.1 mmol/L, or use of anti-diabetic medication. Ethnicity was genetically determined and divided into two different categories: European and other ethnicities.

Total plasma homocysteine level was determined with high-performance liquid chromatography. The CRP levels were measured by kinetic nephelometry.

Statistical analysis

Medication use involved anytime use of antacids (H2 receptor antagonists, prostaglandins, PPIs, combinations of drugs for eradication of *H. pylori* and/or other anti-acids). Also, anytime use of *H. pylori* sensitive antibiotics were examined, which include clarithromycin,

amoxicillin and tetracycline. Anytime use involved medication use at baseline or during follow-up of the study.

Missing covariate data (maximum 11.2%) were imputed using 5-fold multiple imputation, based on included covariates. Distribution of covariates was similar in the imputed versus non-imputed dataset.

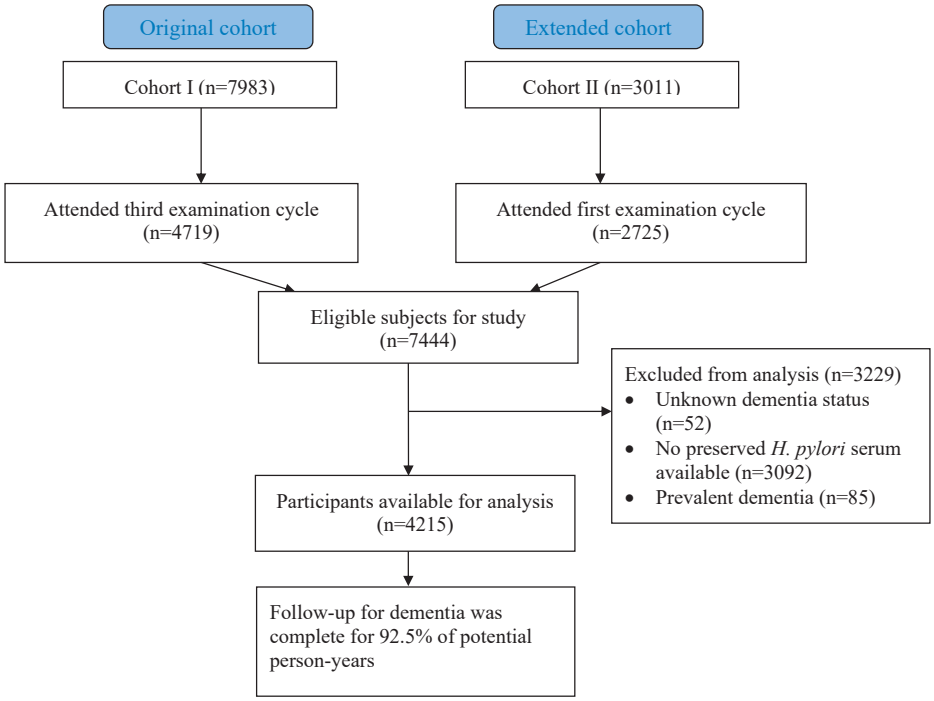

Figure S1. Flow-chart of study population

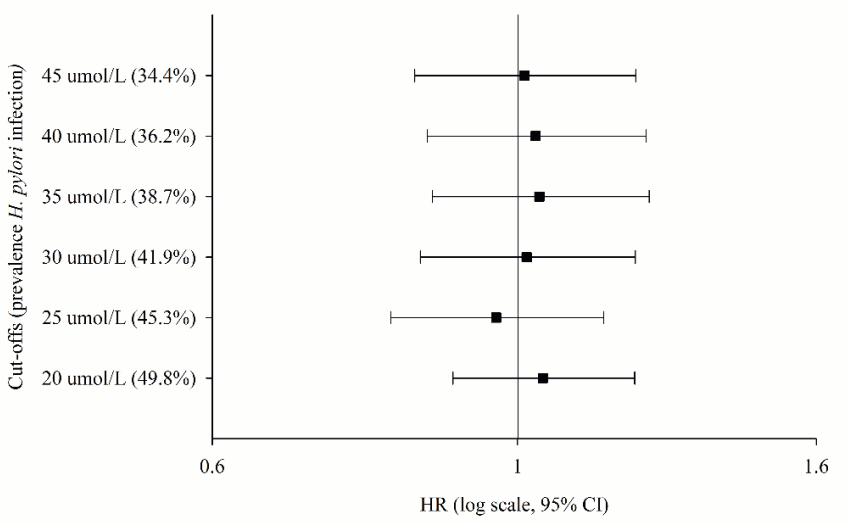

Figure S2. Sensitivity analyses for cut-off values for anti-Helicobacter pylori serum IgG antibody titers for obtaining seroprevalence states.

Abbreviations: H. pylori, helicobacter pylori; U/mL, units of activity per millilitre; HR, hazard ratio. Sensitivity analyses for the effect of *H. pylori* serum IgG (per log increase) on the dementia-free survival of persons in a Cox regression model with the following covariates: cohort, sex, age, education, smoking, blood pressure, anti-hypertensive drug use, body mass index, cholesterol, high-density lipoprotein cholesterol, triglycerides, APOEε carrier status, stroke, diabetes mellitus, ethnicity and serum lipid lowering agents. Error bars indicate 95% confidence intervals of the HR.

Number of persons at risk = 4215, number of cases = 529.

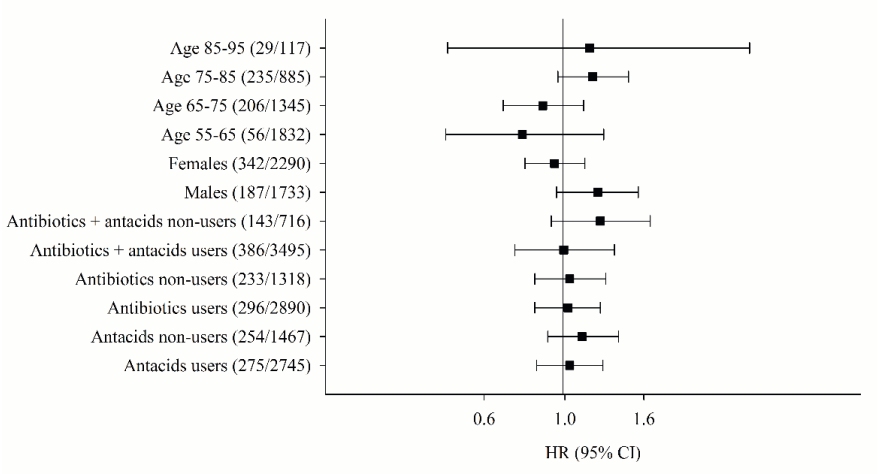

Figure S3. Sensitivity analyses for age, gender and medication use on the association between anti-Helicobacter pylori serum IgG antibody titers and the risk of dementia.

Abbreviations: n = number of cases; N = total number at risk; H. pylori = helicobacter pylori; PPI = proton pump inhibitor; U/mL = units of activity per millilitre; HR = hazard ratio; histamine-2 receptor antagonists = H2 antagonists.

Sensitivity analyses for the effect of *H. pylori* serum IgG antibody titers per log increase on the dementia-free survival of persons in a fully adjusted Cox regression model stratified for the following: age, gender and anytime use of medication (n/N). Medication use includes use of antibiotics (tetracycline, clarithromycin and/or amoxicillin) and antacids (H2 receptor antagonists, prostaglandins, PPIs, combinations of drugs for eradication of *H. pylori* and/or other anti-acids). Error bars indicate 95% confidence intervals of the HR.

Chapter 3
Immunity and stroke

3.1 Subclinical atherosclerosis, and cardiovascular disease

ABSTRACT

Background: Atherosclerotic cardiovascular disease (ASCVD) is driven by multifaceted contributions of the immune system. However, the dysregulation of immune cells that leads to ASCVD is poorly understood. We determined the association of components of innate and adaptive immunity longitudinally with ASCVD, and assessed whether arterial calcifications play a role in this association.

Methods and findings: Granulocyte (innate immunity) and lymphocyte (adaptive immunity) counts were determined 3 times (2002–2008, mean age 65.2 years; 2009–2013, mean age 69.0 years; and 2014–2015, mean age 78.5 years) in participants of the population-based Rotterdam Study without ASCVD at baseline. Participants were followed-up for ASCVD or death until 1 January 2015. A random sample of 2,366 underwent computed tomography at baseline to quantify arterial calcification volume in 4 vessel beds. We studied the association between immunity components with risk of ASCVD and assessed whether immunity components were related to arterial calcifications at baseline. Of 7,730 participants (59.4% women), 801 developed ASCVD during a median follow-up of 8.1 years. Having an increased granulocyte count increased ASCVD risk (adjusted hazard ratio for doubled granulocyte count [95% CI] = 1.78 [1.34–2.37], $P < 0.001$). Higher granulocyte counts were related to larger calcification volumes in all vessels, most prominently in the coronary arteries (mean difference in calcium volume [mm^3] per SD increase in granulocyte count [95% CI] = 32.3 [9.9–54.7], $P < 0.001$). Respectively, the association between granulocyte count and incident coronary heart disease and stroke was partly mediated by coronary artery calcification (overall proportion mediated [95% CI] = 19.0% [–10% to 32.3%], $P = 0.08$) and intracranial artery calcification (14.9% [–10.9% to 19.1%], $P = 0.05$). A limitation of our study is that studying the etiology of ASCVD remains difficult within an epidemiological setting due to the limited availability of surrogates for innate and especially adaptive immunity.

Conclusions: In this study, we found that an increased granulocyte count was associated with a higher risk of ASCVD, whereas an increase in lymphocytes was protective for ASCVD in the general population. Moreover, higher levels of granulocytes were associated with larger volumes of arterial calcification. Arterial calcifications may explain a proportion of the link between granulocytes and ASCVD.

INTRODUCTION

Atherosclerotic cardiovascular disease (ASCVD) arises from various interacting pathophysiological processes [1]. Recent findings point towards a key role of the immune system, which can be broadly classified into innate and adaptive immunity [2]. Innate immunity refers to immune responses present at birth, whereas adaptive immunity is acquired during life by exposure to antigens [3]. The role of innate immunity in the pathophysiology of ASCVD has been recognized on the basis of evidence from experimental and observational data [4–8]. To date, however, no easy, accessible, low-cost treatment has been identified to effectively target the innate immune system for preventing ASCVD [8]. Given the mechanistic diversity of inflammatory pathways, considering other pathways could widen avenues for therapeutic targets, for example targeting the adaptive immune system. Adaptive immune cells may provide protective responses at atherosclerotic sites, as has been shown in neurodegenerative disorders [9,10] and also in cardiovascular disease in experimental and clinical studies [11]. Moreover, repeatedly measuring inflammation may better capture the biologically dynamic processes of these 2 immune systems than a single measurement, because of the assessment of changes over time.

Recent work from the field of cancer research suggests that measuring granulocytes and platelets provides important markers of innate immunity [12–14], whereas measuring lymphocytes yields information on adaptive immunity [15]. Furthermore, combining these measurements into ratios, i.e., the granulocyte-to-lymphocyte ratio (GLR), platelet-to-lymphocyte ratio (PLR), and systemic immune-inflammation index (SII), is thought to even better reflect the relative balance between innate and adaptive immunity [16–18]. In this study, we first determined the longitudinal association of these immunity components with risk of ASCVD in the general population by using the framework of joint models for longitudinal and survival data, and hypothesized that an increase in innate immunity markers and decrease in adaptive immunity lead to increased risk of ASCVD. Second, we examined the association between the immunity components and arterial calcifications to explore atherosclerosis as a possible mediator.

METHODS

Study population

The present study is embedded within the Rotterdam Study, a prospective population-based cohort study in Rotterdam, the Netherlands. The Rotterdam Study started in 1989 with 7,983 persons (78% response rate) aged ≥55 years and residing in the district Ommoord, a suburb of Rotterdam. This first subcohort (RS-I) was extended with a second subcohort (RS-II) in 2000, consisting of 3,011 persons (67% response rate) aged ≥55 years, and with a

third subcohort (RS-III) in 2006, composed of 3,932 persons aged ≥45 years (65% response rate). The design of the Rotterdam Study has been described in detail previously [19]. In brief, participants were examined at study entry and at follow-up visits every 3 to 5 years. They were interviewed at home by a trained research nurse, followed by 2 visits at the research facility for additional interviewing, laboratory assessments, and imaging.

The Rotterdam Study has been approved by the Medical Ethics Committee of the Erasmus MC (registration number MEC 02.1015) and by the Dutch Ministry of Health, Welfare and Sport (Population Screening Act [WBO], license number 1071272-159521-PG). The Rotterdam Study personal registration data collection is filed with the Erasmus MC Data Protection Officer under registration number EMC1712001. The Rotterdam Study has been entered into the Netherlands Trial Register (https://www.trialregister.nl) and into the WHO International Clinical Trials Registry Platform (https://www.who.int/ictrp/network/primary/en/) under the shared catalogue number NTR6831. All participants provided written informed consent to participate in the study and to have their information obtained from treating physicians.

For the current study, the analysis plan was drafted in January 2019 (S1 Analysis Plan). This study is reported as per the Strengthening the Reporting of Observational Studies in Epidemiology (STROBE) guideline (S1 STROBE Checklist).

Laboratory tests for granulocytes, platelets, and lymphocytes were introduced from 2002 onwards, corresponding with the following assessment rounds in the Rotterdam Study (baseline in this study): fourth round of RS-I, second round of RS-II, and first round of RS-III (n = 9,996 in total).

Assessment of blood cell counts and their derived ratios

Fasting blood samples were drawn during each visit at the research center, with a maximum of 3 visits during follow-up. From 7,730 participants, 5,085 participants were available for a second blood cell assessment, and 270 participants were available for a third blood cell assessment. Full blood count measurements were performed using the Coulter AcT diff2 Hematology Analyzer (Beckman Coulter, Brea, CA, US) directly after the blood sample was drawn. Laboratory measurements included absolute granulocyte, platelet, and lymphocyte counts in 10^9 per liter. GLR and PLR were calculated as the ratio of granulocyte count to lymphocyte count and as the ratio of platelet count to lymphocyte count, respectively. SII was defined as platelet count times GLR [20].

Assessment of stroke and coronary heart disease

The clinical outcomes of interest included first fatal or nonfatal stroke or coronary heart disease (CHD) separately as well as combined into ASCVD as previously described. Follow-up data were collected through general practitioners and subsequent collection of information from letters by medical specialists and discharge reports, in cases of hospitalization. CHD

included (1) nonfatal myocardial infarction (MI) and (2) CHD mortality (mortality with definite MI, definite CHD, or possible CHD as underlying cause of death) [21]. Stroke was defined as a syndrome of rapidly developing symptoms of focal or global cerebral dysfunction lasting ≥24 hours or leading to death, with apparent vascular cause [22,23]. We categorized strokes as ischemic or hemorrhagic, based on neuroimaging reports and clinical symptoms, or as unspecified if we were unable to differentiate the stroke type further. Subarachnoid hemorrhages due to ruptured aneurysms were not considered as stroke. Follow-up was virtually complete until 1 January 2015 (96.6% of potential person-years observed). ASCVD furthermore included other ASCVD mortality.

Assessment of arterial calcification

Non-contrast computed tomography (CT) images were obtained using a 16-slice or 64-slice multidetector CT scanner (Somatom Sensation 16 or 64; Siemens, Forchheim, Germany). Using a cardiac scan and an extracardiac scan that reached from the aortic root to the intracranial vasculature (1 centimeter above the sella turcica), we visualized the following vessels: coronary arteries, aortic arch, extracranial internal carotid arteries, and intracranial internal carotid arteries. Radiation dose and other imaging parameters of both scans are described elsewhere [24,25].

The amount of calcification in the coronary arteries, aortic arch, and extracranial carotid arteries was quantified using widely used, commercially available dedicated software (Syngo. via; Siemens). Calcification in the coronary arteries comprised a summation of calcification in the left main, left anterior descending, left circumflex, and right coronary artery. Calcification in the aortic arch was scored from the origin of the aortic arch (defined as the image in which the ascending and descending aorta merge into the inner curvature of the aortic arch) to the first 1 cm of the branches originating from the arch. Calcification in the extracranial carotid arteries was assessed within 3 cm proximal and distal of the bifurcation on both sides and summed.

Intracranial internal carotid artery calcification was assessed from the horizontal segment of the petrous internal carotid artery to the top of the internal carotid artery. Scoring was done semi-automatically, because automated software is not yet available for this region (the close relationship between calcification and the skull base precludes the use of commercial software given a high false-positive rate). Regions of interest were manually drawn in the course of the intracranial internal carotid artery on both sides, after which the number of pixels within these regions above 130 Hounsfield units was summed and multiplied by the pixel size and the slice increment in order to obtain a volume in cubic millimeters [26,27].

Covariates

We assessed education, smoking, and use of antihypertensive, lipid-lowering, and antidiabetic medication at baseline by interview. Diastolic and systolic blood pressures were mea-

sured twice on the right arm with a random-zero sphygmomanometer, of which the mean was used. Total cholesterol and high-density lipoprotein (HDL) cholesterol were measured with a Coulter AcT diff2 Hematology Analyzer. Body mass index (BMI) was computed from measurements of height and weight (kg/m^2). Diabetes mellitus was defined as use of antidiabetic medication, fasting serum glucose level ≥ 7.1 mmol/l (≥127.9 mg/dl), or random non-fasting serum glucose level ≥ 11.1 mmol/l (≥200.0 mg/dl) [28]. High-sensitivity C-reactive protein (hs-CRP) was determined in serum that was drawn during the third assessment round in the Rotterdam Study, i.e., third round of RS-I (1996–1999), which was stored at −20 °C until performance of the hs-CRP measurements in 2003 to 2004. Hs-CRP was measured using Rate Near Infrared Particle Immunoassay (Immage Immunochemistry System, Beckman Coulter). History of cancer was obtained from general practitioners' medical records (including hospital discharge letters), the Dutch Hospital Data registry, and regional histopathology and cytopathology registries. Furthermore, we assessed history of immune-modulating medication use—nonsteroidal anti-inflammatory drugs (NSAIDs) (ATC code M01), immunosuppressives (ATC code L04), and methotrexate (ATC code L01BA01)—by number of prescriptions between 1 January 1995 and blood draw date.

Statistical analysis

Because the blood cell counts and their derived ratios have a skewed distribution and to adhere to the linearity assumptions, the analysis was based on the natural logarithmic (Ln) transformation of their values. We first determined the association of the granulocyte, platelet, and lymphocyte counts and their derived ratios with the risk of stroke and CHD separately as well as ASCVD, using the framework of joint models for longitudinal and survival data [29]. For the longitudinal data, a linear mixed effects model was used, in which we specified the immunity component as the dependent variable and time as the independent variable to assess the mean change in immunity components. Random intercepts and slopes were used to incorporate individual response trajectories. For modeling survival data, we used a Cox regression model in which we included the true underlying profile of the blood cell counts as estimated from the longitudinal model. Here the baseline risk function assumed a piecewise constant with 6 knots placed at equally spaced percentiles of the observed event times. Joint models allow accounting for (1) measurement error during follow-up, i.e., biological variation, (2) the effect of factors at an earlier time point on the values of blood cell measurements at a later time point [30], and (3) the correlations in the repeated measurements of the blood cell counts. Because we are using log-log models ($\log Y_i = \alpha + \beta \log X_i + \varepsilon$), we report hazard ratios (HRs) with 95% confidence intervals (CIs) obtained by exponentiating Ln(2) times the estimated coefficients from the Cox model. These HRs correspond to the increase in risk for a doubling of the blood cell count or ratio. We computed 2 nested models: Model I was adjusted for baseline age (continuous, centered as age minus mean age) and sex; model II was additionally adjusted for education, smoking

status, BMI, diabetes mellitus, systolic and diastolic blood pressure (continuous, centered as blood pressure minus mean blood pressure), antihypertensive medication, total cholesterol, HDL cholesterol, and lipid-lowering medication. For assessment of the association between individual blood cell counts and ASCVD, we performed the analyses with adjustment for the baseline blood cell counts of the remaining 2 blood cell types. Follow-up time was used as the time scale and started at the date of first laboratory assessment; follow-up continued until date of stroke, CHD, death, loss to follow-up, or 1 January 2015, whichever came first. We also investigated the association of the blood cell counts and their ratios with ischemic and hemorrhagic stroke separately. We assessed the joint model assumptions by examining the proportional hazards assumption using Schoenfeld residuals, and goodness of fit using Cox–Snell residuals for the survival part and marginal residuals for the longitudinal part.

In sensitivity analyses, we additionally excluded participants with a history of cancer or a history of immune-modulating medication at baseline. In addition, we performed additional adjustments for NSAID use. We explored effect modification by stratifying by age, sex, hs-CRP (at a cutoff of 2 mg/l), use of lipid-lowering medication, coronary artery calcification volume (at a cutoff of 10 mm^3), and smoking, all at baseline. We formally tested interaction between these factors and blood cell counts on the multiplicative scale by adding interaction terms to model II. Lastly, we repeated the analyses for the granulocytes and lymphocytes and risk of ASCVD using age as the time scale instead of follow-up time to account for potential residual confounding by age and to minimize potential effects of left truncation.

Next, in order to explore possible mechanisms, we performed a cross-sectional analysis assessing the association of the granulocyte, platelet, and lymphocyte counts and their derived ratios at baseline, per standard deviation increase, with calcification volume in each vessel bed (coronary arteries, aortic arch, extracranial carotid arteries, and intracranial carotid arteries) using linear regression models. To facilitate interpretation of these analyses, we chose to first standardize the blood cell counts only, to make results comparable, and keep the rest of the variables untransformed. We adjusted these analyses for age and sex (model I), and additionally for education, smoking status, BMI, diabetes mellitus, systolic blood pressure, diastolic blood pressure, antihypertensive medication, total cholesterol, HDL cholesterol, and lipid-lowering medication (model II). To adhere to the homoscedasticity and linearity assumptions, we repeated linear regression models after natural log-transforming exposure and outcome variables. As a sensitivity analysis, we looked at the association between the granulocyte, platelet, and lymphocyte counts and their derived ratios and continuous arterial calcification only in those with non-zero arterial calcification, since the magnitude of the increase from 0 to 1 for volume may not be the same as other incremental increases in arterial calcification.

Finally, we performed a mediation analysis to assess a possible mediating effect of calcification volume in the coronary arteries and intracranial carotid arteries on the association between innate immunity and the risk of CHD and stroke, respectively. We chose the innate

immunity marker displaying the strongest association with both calcifications and incident CHD or stroke for this analysis. We tested the overall proportion mediated by calcifications. To test for mediation effects, we used the decomposition analysis techniques as described by VanderWeele [31]. Participants with a history of CHD or stroke were excluded from the analysis. We analyzed the association of innate immunity with calcifications with linear regression models. The association of innate immunity or calcifications with CHD or stroke was investigated by Cox regression models.

Multiple imputation by chained equations was used for missing covariates (maximum of 1.5%), with 5 imputed datasets based on other covariates and the outcome. Rubin's method was used for pooling the 5 analyses to obtain pooled HRs and 95% CIs [32]. Two-sided $P < 0.05$ was considered statistically significant for all analyses. Statistical analyses were performed using the R packages *mice, survival, nlme, JM,* Results

From 8,712 participants, we excluded those with a history of ASCVD (n = 819) at baseline, those who had incomplete data regarding their history of ASCVD (n = 95), and those who did not provide informed consent to assess medical records during follow-up (n = 60). Lastly, we excluded participants with incomplete laboratory assessment (n = 8), resulting in 7,730 participants for analysis (flowchart in Fig 1). From the fourth round of RS-I and the second round of RS-II, a random sample was invited to undergo non-contrast CT to quantify arterial calcification. Of these participants, 2,366 had complete calcification and blood assessment.

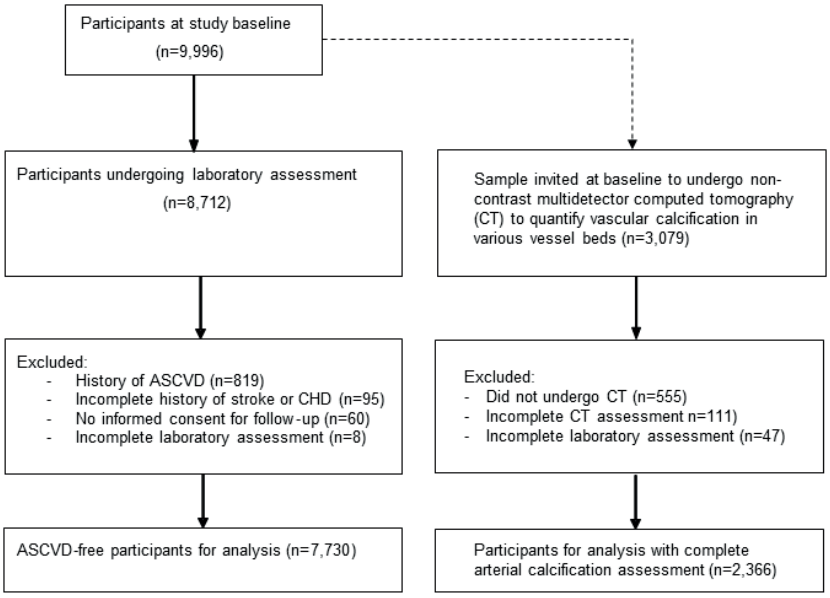

Fig 1. Flowchart of study population.
ASCVD, atherosclerotic cardiovascular disease; CHD, coronary heart disease.

Characteristics of the study participants are presented in Table 1. Mean age of included study participants was 65.2 years, and 59.4% were women. During a median (interquartile range) follow-up of 8.1 (3.4) years (62,095 person-years), 801 participants developed ASCVD, of whom 423 participants developed CHD and 378 stroke. ASCVD-free survival probability and average change of granulocytes and lymphocytes over time are presented in S1 Fig in the total population and in S2 Fig in younger and older individuals separately.

Table 1. Baseline characteristics.

Characteristic	Laboratory assessment cohort N = 7,730	Sample undergoing CT N = 2,366
Women	4,594 (59.4%)	1,236 (52.2%)
Age, years	65.2 (10.2)	69.1 (6.7)
Smoking		
Ever	5,278 (68.7%)	1,662 (71.2%)
Never	2,401 (31.3%)	670 (28.7%)
Diabetes mellitus	415 (5.4%)	129 (5.5%)
BMI, kg/m^2	27.6 (4.4)	27.6 (4.0)
Education		
Primary	830 (10.8%)	179 (7.7%)
Lower	3,124 (40.7%)	979 (42.1%)
Intermediate	2,205 (28.8%)	716 (30.8%)
Higher	1,509 (19.7%)	451 (19.4%)
Systolic blood pressure, mm Hg	142.3 (21.8)	146.7 (20.0)
Diastolic blood pressure, mm Hg	81.3 (11.0)	80.2 (10.7)
Antihypertensive drugs	2,571 (33.6%)	921 (39.5%)
Total cholesterol, mmol/l	5.7 (1.0)	5.6 (1.0)
HDL cholesterol, mmol/l	1.5 (0.4)	1.4 (0.4)
Lipid-lowering medication	1,429 (18.7%)	535 (23.0%)
Granulocyte count, ×10^3/µl	4.0 (1.4)	4.0 (1.3)
Platelet count, ×10^3/µl	270.9 (66.6)	259.0 (64.0)
Lymphocyte count, ×10^3/µl	2.3 (1.1)	2.3 (1.0)
GLR	1.9 (0.8)	1.9 (0.9)
PLR	127.9 (46.4)	124.9 (45.8)
SII	509.3 (270.7)	494.3 (272.2)

Data presented as mean (standard deviation) for continuous variables and number (percentage) for categorical variables. Number of missing values for the ASCVD-free cohort: 51 (0.7%) for smoking, 22 (0.3%) for diabetes, 25 (0.3%) for BMI, 62 (0.8%) for education, 41 for systolic and diastolic blood pressure (0.5%), 69 for blood pressure lowering medication (0.9%), 25 for cholesterol (0.3%), 27 for HDL cholesterol (0.3%), and 69 for lipid lowering medication (0.9%). Number of missing values for the sample undergoing CT are: 52 (0.6%) for smoking, 22 (0.3%) for diabetes, 26 (0.3%) for BMI, 41 (0.5%) for education, 14 for systolic and diastolic blood pressure (0.6%), 35 for blood pressure lowering medication (1.5%), 6 for cholesterol (0.3%), 6 for HDL cholesterol (0.3%) and 35 for lipid lowering medication (1.5%).

BMI, body mass index; CT, computed tomography; GLR, granulocyte-to-lymphocyte ratio; PLR, platelet-to-lymphocyte ratio; SII, systemic immune-inflammation index.

An increased granulocyte count, but not platelet count, was associated with an increased risk of ASCVD (adjusted HR [95% CI] for doubled granulocyte count = 1.78 [1.34–2.37], P < 0.001, and for doubled platelet count = 1.17 [0.90–1.51], P = 0.24, Table 2). With respect to adaptive immunity, an increased level of lymphocytes was associated with a decreased risk of ASCVD, although this result was not statistically significant (adjusted HR for doubled lymphocyte count [95% CI] = 0.87 [0.71–1.06], P = 0.17). Both an increased GLR and an increased SII were associated with increased ASCVD risk (adjusted HR for doubled GLR [95% CI] = 1. 37 [1.14–1.65], P = 0.001, and for doubled SII = 1.19 [1.03–1.36], P = 0.02), whereas an increased PLR was not associated with ASCVD risk. We observed no major violations of the joint model assumptions. Effect estimates did not materially change, and in fact became stronger, when we excluded participants with a history of cancer or history of immune-modulating medication at baseline (723 ASCVD cases among 7,063 remaining participants) (adjusted HR for doubled granulocyte count [95% CI] = 1.90 [1.41–2.57], P < 0.001, and for doubled lymphocyte count = 0.82 [0.65–1.04], P = 0.10). Additional adjustment for NSAID use did not change the effect estimates. We found that the association between higher levels of granulocytes and risk of ASCVD was more pronounced in participants aged younger than the median age of 64 years compared to those 64 years or older (adjusted HR for doubled granulocyte count [95% CI] = 2.32 [1.20–4.47], P < 0.001) ($P_{interaction}$ = 0.002). Results of all stratified analyses with interaction terms are shown in S1 Table. Lastly, risk estimates were comparable when using age as the time scale instead of follow-up time.

Table 2. Joint models for the association between repeated blood-based immunity markers and subsequent risk of ASCVD.

Model and immunity marker	ASCVD n/N = 801/7,730		Stroke n/N = 378/7,730		CHD n/N = 423/7,730	
	HR (95% CI)	**P value**	**HR** (95% CI)	**P value**	**HR** (95% CI)	**P value**
Model I						
Innate immunity*						
Granulocytes	2.63 (2.03–3.40)	<0.001	2.14 (1.48–3.11)	<0.001	3.10 (2.18–4.42)	<0.001
Platelets	1.10 (0.85–1.41)	0.47	1.27 (0.88–1.83)	0.21	0.96 (0.68–1.36)	0.83
Adaptive immunity*						
Lymphocytes	1.03 (0.85–1.25)	0.77	0.96 (0.72–1.29)	0.80	1.09 (0.83–1.43)	0.54
Balance between innate and adaptive immunity						
GLR	1.52 (1.27–1.82)	<0.001	1.47 (1.13–1.91)	0.004	1.56 (1.22–2.01)	<0.001
PLR	1.02 (0.87–1.19)	0.84	1.15 (0.91–1.46)	0.24	0.97 (0.78–1.20)	0.76
SII	1.25 (1.09–1.43)	0.002	1.27 (1.04–1.56)	0.02	1.24 (1.03–1.51)	0.03

Table 2. Joint models for the association between repeated blood-based immunity markers and subsequent risk of ASCVD. (continued)

Model and immunity marker	ASCVD n/N = 801/7,730		Stroke n/N = 378/7,730		CHD n/N = 423/7,730	
	HR (95% CI)	**P value**	**HR** (95% CI)	**P value**	**HR** (95% CI)	**P value**
Model II						
Innate immunity*						
Granulocytes	1.78 (1.34–2.37)	<0.001	1.50 (1.00–2.27)	0.05	1.98 (1.34–2.93)	0.001
Platelets	1.17 (0.90–1.51)	0.24	1.38 (0.95–2.01)	0.09	1.01 (0.71–1.44)	0.95
Adaptive immunity*						
Lymphocytes	0.87 (0.71–1.06)	0.17	0.85 (0.63–1.15)	0.29	0.88 (0.66–1.17)	0.37
Balance between innate and adaptive immunity						
GLR	1.37 (1.14–1.65)	0.001	1.31 (1.00–1.70)	0.05	1.37 (1.07–1.78)	0.01
PLR	1.17 (0.99–1.38)	0.06	1.25 (0.98–1.59)	0.07	1.10 (0.88–1.38)	0.41
SII	1.19 (1.03–1.36)	0.02	1.21 (0.99–1.48)	0.06	1.16 (0.96–1.41)	0.13

HR is per doubled blood cell count. Model I is adjusted for age and sex. Model II is adjusted for age, sex, education, smoking status, body mass index, diabetes mellitus, systolic and diastolic blood pressure, antihypertensive medication, high-density lipoprotein cholesterol, total cholesterol, and lipid-lowering medication=. All markers were logarithmically transformed.

*Analysis for each blood cell type adjusted for the baseline blood cell counts of the remaining 2 blood cell types.

ASCVD, atherosclerotic cardiovascular disease; CHD, coronary heart disease; CI, confidence interval; GLR, granulocyte-to-lymphocyte ratio; HR, hazard ratio; *n,* number of incident events; *N,* number of participants for analysis; PLR, platelet-to-lymphocyte ratio; SII, systemic immune-inflammation index (defined as platelet count times GLR).

When examining CHD and stroke as separate events, an increased granulocyte count was associated with an increased risk of both events (adjusted HR for doubled granulocyte count [95% CI] = 1.98 [1.34–2.93], *P* = 0.001, for CHD and 1.50 [1.00–2.27], *P* = 0.05, for stroke; Table 2). An increased platelet count was associated with higher risk of stroke only (adjusted HR for doubled platelet count [95% CI] = 1.38 [0.95–2.01], *P* = 0.09). An increased lymphocyte count showed similar protective effects for both CHD and stroke, although these effects were not statistically significant (adjusted HR for doubled lymphocyte count [95% CI] = 0.88 [0.66–1.17], *P* = 0.37, for CHD and 0.85 [0.63–1.15], *P* = 0.29, for stroke). With respect to the ratio measures, an increased GLR was associated with both increased CHD and stroke risk (adjusted HR for doubled GLR [95% CI] = 1.37 [1.07–1.78], *P* = 0.01, for CHD and 1.31 [1.00–1.70], *P* = 0.05, for stroke). When investigating ischemic and hemorrhagic stroke separately, an increased granulocyte count

was associated with an increased risk of ischemic stroke (HR for doubled granulocyte count [95% CI] = 1.44 [0.90–2.31], P = 0.13) and hemorrhagic stroke (2.48 [0.86–7.14], P = 0.09), whereas platelets only increased risk of ischemic stroke (HR for doubled platelet count [95% CI] = 1.44 [0.93–2.22], P = 0.10).

Regarding the association of immunity with arterial calcification, we found that higher levels of granulocytes at baseline related to larger calcification volumes in all arteries, but most prominently in the coronary arteries (mean difference in calcium volume [mm^3] per SD increase in granulocyte count [95% CI] = 32.3 [9.1 to 54.7], P < 0.001). Regarding adaptive immunity, higher levels of lymphocytes were not related to calcification volumes. Higher levels of GLR and SII were related to larger calcification volumes in the aortic arch only (mean difference in calcium volume [mm^3] per SD increase [95% CI] = 48.6 [2.5 to 94.8], P = 0.04, and 58.8 [13.8 to103.8], P = 0.03, respectively). In Table 3, the linear regression models after transforming the exposure and outcome variables are presented. Our sensitivity analyses with non-zero calcifications similarly showed associations between higher levels of granulocytes and larger calcification volumes in all arteries, but the analysis with extracranial internal carotid artery calcification as outcome did not reach statistical significance.

Table 3. Models for the association between blood-based immunity markers and arterial calcification volume.

Immunity marker[a]	Arterial calcification volume[b]							
	Coronary arteries		Aortic arch		Extracranial internal carotid		Intracranial internal carotid	
	Mean difference (95% CI)	P value	Mean difference (95% CI)	P value	Mean difference (95% CI)	P value	Mean difference (95% CI)	P value
Innate immunity*								
Granulocytes	0.26 (0.14; 0.39)	<0.001	0.20 (0.07; 0.32)	<0.001	0.22 (0.09; 0.35)	<0.001	0.18 (0.05; 0.32)	0.01
Platelets	−0.02 (−0.18; 0.13)	0.76	0.01 (−0.14; 0.17)	0.87	−0.05 (−0.21; 0.11)	0.57	−0.09 (−0.25; 0.07)	0.27
Adaptive immunity*								
Lymphocytes	0.15 (0.03; 0.27)	0.02	0.01 (−0.11; 0.13)	0.91	0.09 (−0.04; 0.21)	0.17	0.08 (−0.04; 0.21)	0.19
Balance between innate and adaptive immunity								
GLR	0.06 (−0.04; 0.15)	0.24	0.10 (0.00; 0.19)	0.04	0.06 (−0.03; 0.16)	0.18	0.04 (−0.05; 0.14)	0.36
PLR	−0.09 (−0.19; 0.01)	0.08	0.01 (−0.09; 0.11)	0.85	−0.06 (−0.17; 0.04)	0.25	−0.08 (−0.18; 0.03)	0.15
SII	0.06 (−0.01; 0.14)	0.10	0.09 (0.01; 0.16)	0.03	0.06 (−0.02; 0.13)	0.15	0.03 (−0.05; 0.11)	0.48

Adjusted for age, sex, education, smoking status, body mass index, diabetes mellitus, systolic and diastolic blood pressure, antihypertensive medication, high-density lipoprotein cholesterol, total cholesterol, and lipid-lowering medication.

[a]All markers were natural log-transformed (Ln[immunity component × 10^3/µl]).

[b]We added 1.0 mm^3 to the non-transformed values to deal with calcification volumes of 0 and used standardized natural log-transformed values (Ln[calcification volume + 1.0 mm^3]).

*Analysis for each blood cell type adjusted for the baseline blood cell counts of the remaining 2 blood cell types.

CI, confidence interval; GLR, granulocyte-to-lymphocyte ratio; PLR, platelet-to-lymphocyte ratio; SII, systemic immune-inflammation index.

The association between granulocytes and incident CHD was partly mediated by coronary artery calcifications (overall proportion mediated [95% CI] = 19.0% [−10% to 32.3%], P = 0.08), while the association between granulocytes and incident stroke was partly mediated by intracranial artery calcifications (14.9% [−10.9% to 19.1%], P = 0.05), albeit this finding was not statistically significant.

DISCUSSION

In this study, we found that an increase of innate immunity markers over time, as reflected by a doubling of the granulocyte count and the ratios GLR and SII, was robustly associated with a higher risk of ASCVD. Furthermore, higher levels of granulocytes at baseline were related to larger burden of subclinical atherosclerosis, as measured by arterial calcifications. Coronary artery calcifications mediated 19.0% of the association between granulocytes and CHD, while intracranial artery calcifications mediated 14.9% of the association between granulocytes and stroke.

To date, few population-based studies have investigated the association between innate immunity markers and cardiovascular outcomes. Elevated GLR has been linked to ischemic stroke incidence and cardiovascular disease [35,36]. A meta-analysis of 38 studies showed that elevated GLR was associated with coronary artery disease, acute coronary syndrome, stroke, and composite cardiovascular events [37]. However, most of these studies were cross-sectional or were performed in selected clinical populations of patients with cardiovascular disease. Besides observational studies, various clinical trials have targeted the immune system to reduce the risk of ASCVD. One of the largest is the CANTOS (Canakinumab Anti-inflammatory Thrombosis Outcome Study) randomized trial, in which patients with elevated hs-CRP and previous MI were assigned to receive canakinumab or placebo. Canakinumab selectively inhibits interleukin-1β, resulting in an inhibition of the innate immunity. The 150-mg dose resulted in a lower incidence of recurrent cardiovascular events, compared to placebo [38]. In addition to interleukin-1β, also hs-CRP, fibrinogen, and interleukin-6, all markers in the same cascade, have been shown to have an association with CHD [39–41]. Our findings also show an important role of innate immunity in first-ever stroke and CHD risk in the general population, independent of hs-CRP level or lipid-lowering medication use.

Interestingly, a more recent trial assessing low-dose methotrexate to reduce inflammation in order to prevent recurrent cardiovascular disease showed that methotrexate did not lower levels of interleukin-1β, interleukin-6, or hs-CRP, and that it did not result in a lower number of cardiovascular events compared to placebo [8]. Together with the results from the CANTOS trial, these studies indicate that reducing the risk of cardiovascular events through inflammation may depend on the pathway targeted. In addition to these established

pathways, our results indicate that the pathway of adaptive immunity may also affect the risk of cardiovascular disease. More specifically, lymphocyte count, an important player in adaptive immunity, showed protective effects on the risk of ASCVD, although this finding was not statistically significant. Indeed, mobilizing anti-inflammatory T cells to atherosclerotic sites may provide protective responses as has been shown in neurodegenerative disorders [9] and also in cardiovascular disease in experimental and clinical studies [11]. These cells thus additionally serve as a promising therapeutic target to control the proinflammatory processes of atherosclerosis. The significant association we found between GLR and SII and incident ASCVD, although weaker than with granulocytes, supports the hypothesis that ASCVD results from an imbalance between innate and adaptive immunity. Future studies may focus more specifically on targeting both innate and adaptive immunity, rather than only innate immunity, and refine insights into these pathways.

Although previous studies have established the inflammatory hypothesis of atherothrombosis in coronary artery disease, fewer studies have specifically investigated stroke. However, increasing evidence from experimental studies suggests an equally important role for the immune response in stroke [42]. Similarly, we demonstrated that higher levels of granulocytes were associated with stroke of any type, as well as with ischemic and hemorrhagic stroke separately. Especially the latter finding warrants further research since most studies so far have mainly focused on inflammatory responses after the occurrence of an intracerebral hemorrhage, and not before [43].

An interesting finding of our study is that the association between higher levels of granulocytes and risk of ASCVD was more pronounced in younger individuals than older individuals (age below the median [64 years]: adjusted HR [95% CI] for doubled granulocyte count = 2.32 [1.20–4.47], $P < 0.001$; age at or above the median: 1.67 [1.21–2.29], $P = 0.002$). Recently, an English and Dutch study found that while there has been a decline in mortality from acute stroke within 30 days, stroke event rates have increased and 15-year mortality risk in young stroke-survivors remained elevated [44,45]. This suggests that stroke awareness and stroke prevention need to start at much lower age to reduce the occurrence of stroke in younger people. Our results show that inflammation reduction could be a potential important strategy to achieve this.

Moreover, our study showed that coronary and intracranial carotid artery calcifications substantially mediate the association of granulocytes with CHD and stroke, respectively. The observed proportion of mediation for CHD was 19.0%, while for stroke the proportion mediated was 14.9%. This is meaningful given the multiple mechanisms through which innate immunity affects cardiovascular health, including atrial fibrillation (AF) and coagulation [46]. Indeed, the infiltration of immune cells and proteins that cause an inflammatory response in cardiac tissue and circulatory processes is associated with AF [47], and AF is in turn an important risk factor for CHD and stroke. Another possible mediator is blood coagulation, since inflammation results in activation of coagulation, due to tissue-factor-

mediated thrombin generation, downregulation of physiological anticoagulant mechanisms, and inhibition of fibrinolysis, increasing the risk of CHD and stroke [48]. Furthermore, besides calcifications, plaques may consist of intraplaque hemorrhage and a lipid core. Although calcifications are an adequate quantitative measure for atherosclerosis, vulnerable plaques without calcification, which are more strongly associated with ASCVD [49], are not quantified by this method. Therefore, it is plausible that the true proportion of inflammation mediated through atherosclerosis of all plaque components is actually higher than the measured proportion mediated through the calcification component.

Blood cell count measurements display short-term variability or measurement error, but we do not expect that this variability affected our results for several reasons. First, we expect to have captured a more chronic or stable effect of immune status because we used blood cell counts measured at multiple time points per participant. Although this longitudinal information was collected intermittently and with error, we could successfully reconstruct the complete longitudinal history by postulating a suitable mixed effects model to describe participant-specific time evolutions. The mixed model accounted for the measurement error problem by postulating that the observed level of the blood cell count measurements equaled the true level of the blood cell count measurements plus a random error term. Thus, the joint models we used accounted for measurement error or variability of the blood cell count measurements. Second, as a sensitivity analysis, we additionally excluded participants with a history of cancer or a history of immune-modulating medication at baseline, and we performed additional adjustments for NSAID use. We performed these sensitivity analyses specifically to address the issue of participants having significantly altered blood cell counts due to underlying disease processes. The results of these analyses did not show significant changes in our effect estimates. Lastly, by extensively correcting for potential confounders such as BMI, we attempted to rule out effects of determinants of blood cell counts as much as possible.

Our study has some limitations. First, studying the etiology of ASCVD, which is the focus of this study, remains difficult within an epidemiological setting due to the limited availability of surrogates for innate and especially adaptive immunity. Second, we could not directly relate the effect of hs-CRP with the different blood cell measurements and ASCVD risk since we did not have hs-CRP measurements available at study baseline, but only 1 round before. Third, the vast majority of our population is of European ancestry (97.6%), potentially limiting generalizability to other ethnicities. Fourth, it is important to note the possible influence of unmeasured confounders on our results. Fifth, due to the cross-sectional nature of the analyses regarding the association between immunity markers and calcifications, there is potential for reverse causality. However, according to the present evidence, it is very plausible that inflammation comes first since activation of endothelial cells results in increased expression of leukocyte adhesion molecules and the release of chemokines, which attract leukocytes that subsequently infiltrate the vascular wall and drive atherogenesis [50].

Finally, the measurement error for both subclinical ASCVD and immune cell subsets limits the mediation analysis in its reliability. However, since this measurement error introduces random error into the direct effect, it is expected to lead to underestimation of the mediation. Of course, we cannot rule out that it could bias the results in the opposite direction and inflate mediation estimates.

In conclusion, our study shows that increased granulocyte count, GLR, and SII are associated with a higher risk of ASCVD, whereas increased lymphocyte count is protective for ASCVD in the general population. Moreover, higher levels of granulocytes are associated with subclinical atherosclerosis, as measured by arterial calcifications. These calcifications explain a substantial proportion of the link between granulocytes and ASCVD. Our findings support the hypothesis that ASCVD results from an imbalance between innate and adaptive immunity.

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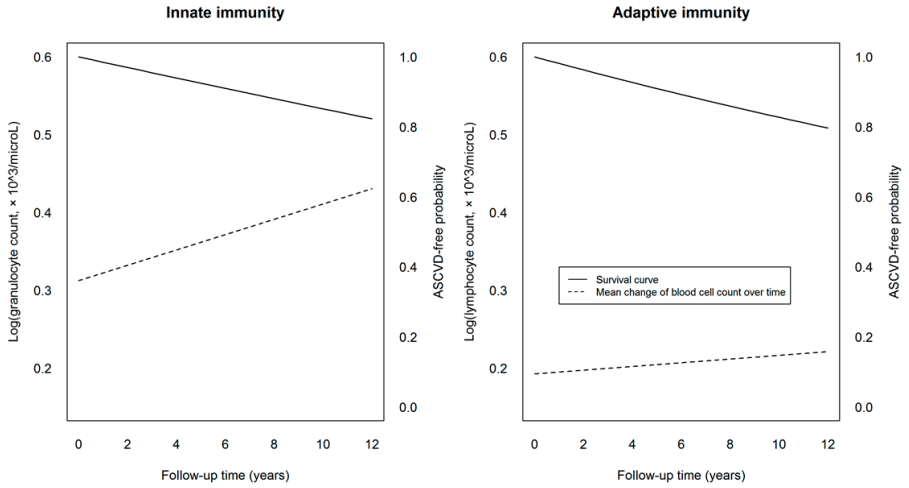
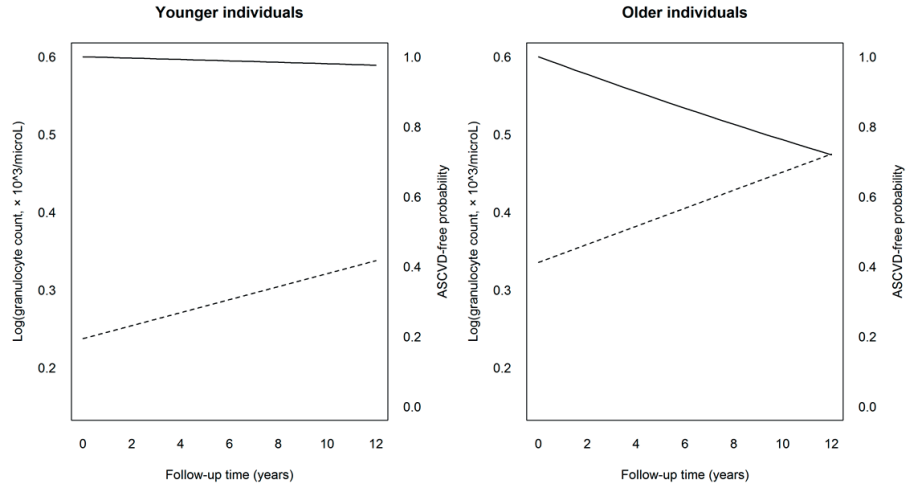
SUPPORTING INFORMATION



S1 Fig. ASCVD-free survival and mean change of blood cell count over time in the total population. The left axis in each panel denotes the blood cell count (dotted line), the right axis the ASCVD-free survival (solid line). All curves were adjusted for all covariates in model II.



S2 Fig. ASCVD-free survival and mean change of granulocyte count over time stratified by younger and older participants.

Younger participants are those below the median age of 64 years. Of these participants, 142 developed ASCVD, out of 3,799 persons at risk.

S1 Table. Models for the stratified analyses.

Laboratory assessmenta		Age		Sex		Smoking
Per doubling	P for interaction	HR (95% CI), P	P for interaction	HR (95% CI), P	P for interaction	
Granulocytes*	0.002	Low: 2.32 (1.20-4.47), <.001	0.245	Women: 1.30 (0.88-1.93), 0.18	0.565	Never: 1.49 (1.06-2.09), 0.02
		High: 1.67 (1.21-2.29), 0.002		Men: 2.32 (1.53-3.52), <.001		Ever: 2.19 (1.27-3.77), 0.005
Platelets*	0.867	Low: 1.65 (0.87-3.14), 0.13	0.167	Women: 1.11 (0.76-1.62), 0.6	0.784	Never: 1.34 (1.00-1.81), 0.05
		High: 1.08 (0.81-1.45), 0.6		Men: 1.26 (0.88-1.79), 0.21		Ever: 0.79 (0.47-1.31), 0.363
Lymphocytes*	0.694	Low: 0.97 (0.56-1.66), 0.9	0.209	Women: 0.83 (0.62-1.11), 0.2	0.028	Never: 0.78 (0.61-0.99), 0.04
		High: 0.87 (0.7-1.09), 0.23		Men: 0.91 (0.68-1.22), 0.55		Ever: 1.06 (0.73-1.55), 0.746
GLR	0.009	High: 1.3 (1.08-1.58), 0.007	0.945	Men: 1.49 (1.15-1.93), 0.003	0.172	Never: 1.38 (1.12-1.7), 0.003
		Low: 1.52 (0.93-2.48), 0.09		Women: 1.25 (0.96-1.62), 0.1		Ever: 1.28 (0.85-1.93), 0.241
PLR	0.8	High: 1.16 (0.97-1.38), 0.11	0.966	Men: 1.19 (0.95-1.49), 0.13	0.009	Never: 1.34 (1.1-1.62), 0.003
		Low: 1.29 (0.77-1.87), 0.41		Women: 1.17 (0.92-1.49), 0.21		Ever: 0.82 (0.61-1.1), 0.181
SII	0.641	High: 1.14 (0.98-1.32), 0.09	0.601	Men: 1.27 (1.05-1.53), 0.02	0.238	Never: 1.22 (1.04-1.43), 0.016
		Low: 1.40 (0.96-2.04), 0.08		Women: 1.11 (0.90-1.37), 0.32		Ever: 1.06 (0.79-1.43), 0.705
		Calcifications		CRP		Lipid-lowering medication
Granulocytes*	0.559	Low: 3.51 (1.14-10.83), 0.03	0.488	Low: 1.47 (0.8-2.71), 0.21	0.798	Yes: 1.77 (0.89-3.49), 0.10
		High: 1.86 (1.05-3.31), 0.03		High: 0.94 (0.5-1.76), 0.84		No: 1.97 (1.45-2.68), <.001
Platelets*	0.178	Low: 0.56 (0.19-1.65), 0.29	0.806	Low: 1.35 (0.76-2.41), 0.31	0.057	Yes: 0.69 (0.39-1.24), 0.21
		High 1.11 (0.68-1.81), 0.69		High: 1.07 (0.62-1.83), 0.82		No: 1.26 (0.95-1.67), 0.12
Lymphocytes*	0.073	Low: 2.22 (1.04-4.71), 0.04	0.176	Low: 0.79 (0.49-1.27), 0.33	0.605	Yes: 0.86 (0.53-1.40), 0.54
		High: 0.73 (0.50-1.09), 0.13		High: 0.79 (0.54-1.16), 0.22		No: 0.93 (0.74-1.16), 0.51

S1 Table. Models for the stratified analyses. (continued)

Laboratory assessmenta		Age		Sex		Smoking
GLR	0.354	Low: 1.03 (0.51-2.08), 0.93	0.925	Low: 1.33 (0.92-1.94), 0.13	0.707	Yes: 1.34 (0.88-2.06), 0.18
		High: 1.55 (1.08-2.24), 0.02		High: 1.15 (0.83-1.59), 0.40		No: 1.41 (1.15-1.74), 0.001
PLR	0.018	Low: 0.54 (0.29-1.02), 0.06	0.45	Low: 1.22 (0.9-1.65), 0.21	0.315	Yes: 0.97 (0.67-1.41), 0.88
		High: 1.29 (0.94-1.77), 0.12		High: 1.22 (0.9-1.65), 0.21		No: 1.17 (0.97-1.41), 0.09
SII	0.176	Low: 0.91 (0.51-1.62), 0.75	0.81	Low: 1.24 (0.92-1.68), 0.16	0.396	Yes: 0.99 (0.72-1.36), 0.94
		High: 1.25 (0.95-1.64), 0.11		High: 1.06 (0.81-1.37), 0.69		No: 1.23 (1.05-1.44), 0.009

Adjusted for age, sex, education, smoking status, body mass index, diabetes mellitus, systolic blood pressure, diastolic blood pressure, antihypertensive medication, HDL cholesterol, total cholesterol, and lipid-lowering medication. [a]All markers were natural log-transformed (Ln[immunity components × 10^3/μl]). *Analysis for each blood cell type adjusted for the baseline blood cell counts of the remaining 2 blood cell types. CI, confidence interval; GLR, granulocyte-to-lymphocyte ratio; PLR, platelet-to-lymphocyte ratio; SII, systemic immune-inflammation index.

Chapter 4
Brain pathology

4.2 Immunity and amyloid-beta, total-tau and neurofilament light chain

ABSTRACT

Introduction

We investigated how components of immunity relate to biomarkers of Alzheimer's disease (AD) in plasma and explored the influence of AD genetic risk factors in the population-based Rotterdam Study.

Methods

In 7,397 persons, we calculated the granulocyte-to-lymphocyte ratio[GLR], platelet-to-lymphocyte ratio[PLR], and systemic immune-inflammation index[SII]. In 3,615 of these persons, plasma amyloid-beta (Aβ)-42 and Aβ-40 were measured. Next, we constructed an overall genetic risk score[GRS] based on genome-wide significant variants, both including and excluding *APOE*-ε4.

Results

All innate immunity phenotypes were related to higher Aβ, most strongly with a doubling in GLR leading to a 1.9% higher Aβ-42 (95% confidence interval [95%CI] 0.4;3.3%) and 3.2% higher Aβ-40 [95%CI 2.0;4.3%]. Higher AD GRS including *APOE*-ε4 was associated with higher immunity markers.

Discussion

Higher levels of immunity markers were associated with higher Aβ in plasma. Participants with a higher genetic predisposition to AD had higher immunity markers, where these effects were mainly driven by *APOE*-ε4.DIntroduction

INTRODUCTION

Alzheimer's disease (AD) is characterized pathologically by accumulation of amyloid-beta (Aβ) as amyloid plaques and phosphorylated tau as neurofibrillary tangles [1]. This accumulation gave rise to the amyloid hypothesis, posing that Aβ activates a cascade of pathologic changes [2]. As an extension to the amyloid hypothesis, the antimicrobial protection model was recently proposed suggesting that Aβ oligomerization is not intrinsically pathological, but emerges as an innate immune response [3]. Genetic variants identified through genome-wide association studies (GWAS) support this role for immunity [4]. It has also been found that higher activation of the innate immune system and lower activation of the adaptive immune system leads to higher dementia risk [5]. Yet, the link between immunity and AD-related brain pathology is largely unknown.

In the early 1990s, Apolipoprotein E (ApoE) was found to be a component of amyloid plaques [6, 7]. However, how ApoE's function as a lipid-carrier is in itself related to AD, either in relation to amyloid-β or other factors, is not entirely clear. Lipids serve more than pure nutritional purposes; they also play essential roles in immune regulation [8]. Innate immunity in turn affects amyloid-β and tau pathology buildup. Insoluble tau isolated from postmortem AD brain is shown to be taken up by microglia in vitro and in vivo [9]. This taking in of tau may participate in tau spreading by microglia subsequently releasing some form of tau [10].

To study further how AD relates to the immune response, we may use serum levels of various blood cells which reflect a systemic immune response [11] and relate them with biomarkers of AD pathology and progression. Serum levels of granulocytes and platelets are known biomarkers of the innate immunity, whereas lymphocyte levels may yield information on the adaptive immunity [12]. Combining these measurements into ratios is thought to even better reflect the relative balance between innate and adaptive immunity, obtaining the granulocyte-to-lymphocyte ratio (GLR), platelet-to-lymphocyte ratio (PLR) and systemic immune-inflammation index (SII) as phenotypical markers of immunity [13]. Whereas for the diagnosis of AD, biomarkers are grouped into those of β amyloid deposition, pathologic tau, and neurodegeneration [ATN] according to the recent classification by Jack et al [14], yielding plasma Aβ, total-tau and neurofilament light chain (NfL) as biomarkers of AD-related brain pathology, respectively.

In an earlier study investigating the role of various biological pathways based on a genetic risk score, we found an important role for the *immune response* pathway in early AD pathology [15]. Relating phenotypical immunity and AD-related markers to each other and additionally to genetic predisposition to AD may help to understand the role of immunity biomarkers in AD pathology and their relation with AD genetic risk, and ultimately identify therapeutic strategies targeting upstream events of altered immune response in amyloidosis and neurodegeneration. We previously found that higher levels of the GLR, PLR and SII

over time reflecting higher innate immunity increase dementia risk [5]. Since we also previously found that low Aβ-42 and high NfL plasma levels were associated with risk of AD [16], we hypothesized that higher innate immunity affects the serum levels of Aβ-42 to be lower and NfL to be higher. We tested this hypothesis by investigating whether serum GLR, PLR and SII were associated with Aβ, total-tau and NfL and how genetic predisposition to AD affected these markers.

METHODS

Study population

The present study is embedded within the Rotterdam Study, a prospective population-based cohort study in Rotterdam, the Netherlands. The Rotterdam Study started in 1989 with 7,983 persons (response of 78%) aged ≥55 years and residing in the district Ommoord, a suburb of Rotterdam. This first subcohort (RS-I) was extended with a second subcohort (RS-II) in 2000, consisting of 3,011 persons (response of 67%) and with a third subcohort (RS-III) in 2006, composed of 3,932 persons aged ≥45 years (response of 65%). The design of the Rotterdam Study has been described in detail previously [17]. In brief, participants were examined at study entry and follow-up visits every three to five years. They were interviewed at home by a trained research nurse, followed by two visits at the research facility for additional interviewing and laboratory assessments. The Rotterdam Study has been approved by the Medical Ethics Committee of the Erasmus Medical Center and by the board of the Netherlands Ministry of Health, Welfare, and Sports. Written informed consent was obtained from all participants.

Laboratory tests for granulocytes, platelets, and lymphocytes were introduced from 2002 onwards, corresponding with the following assessment rounds in the Rotterdam Study (baseline for this study): fourth round of RS-I (RS-I-4), second round of RS-II (RS-II-2), and first round of RS-III (RS-III-1). Plasma biomarkers of AD-related brain pathology were only assessed in RS-I-4 and RS-II-2. Blood was drawn from 11,496 participants for genotyping. Of these participants, 7,413 underwent granulocytes, platelets, and lymphocytes assessments, while 3,627 participants underwent plasma Aβ, total-tau and NfL assessment. We excluded participants with missing *APOE* genotype and participants with incomplete assessments of serum markers, leaving (n=7,397) participants for analysis with complete blood assessment for immunity markers and 3,615 participants with complete plasma Aβ, total-tau and NfL assessment (**Figure 1**).

Genotyping

DNA genotyping was performed at the internal genotyping facility of the Erasmus Medical Center, Rotterdam. All samples were genotyped with the 550K, 550K duo or 610K Illumina

arrays. Genotyping quality control criteria include call rate <95%, Hardy-Weinberg equilibrium of $P < 1.0 \times 10^{-6}$, and minor allele frequency <1%. Moreover, study samples with excess autosomal heterozygosity, call rate <97.5%, ethnic outliers, and duplicate or family relationships were excluded during quality control analysis. Genetic variants were imputed from the Haplotype Reference Consortium reference panel (version 1.0) [18], using the Michigan imputation server [19]. The server uses SHAPEIT2 (v2.r790) [20] to phase the genotype data and performs imputation with Minimac 3 software [21]. For this study, we used genetic variants that had imputation quality (R^2) > 0.5. *APOE* genotype was determined separately by PCR on coded DNA samples in the baseline cohort and with a bi-allelic Tacqman assay in the extensions of the Rotterdam Study [22].

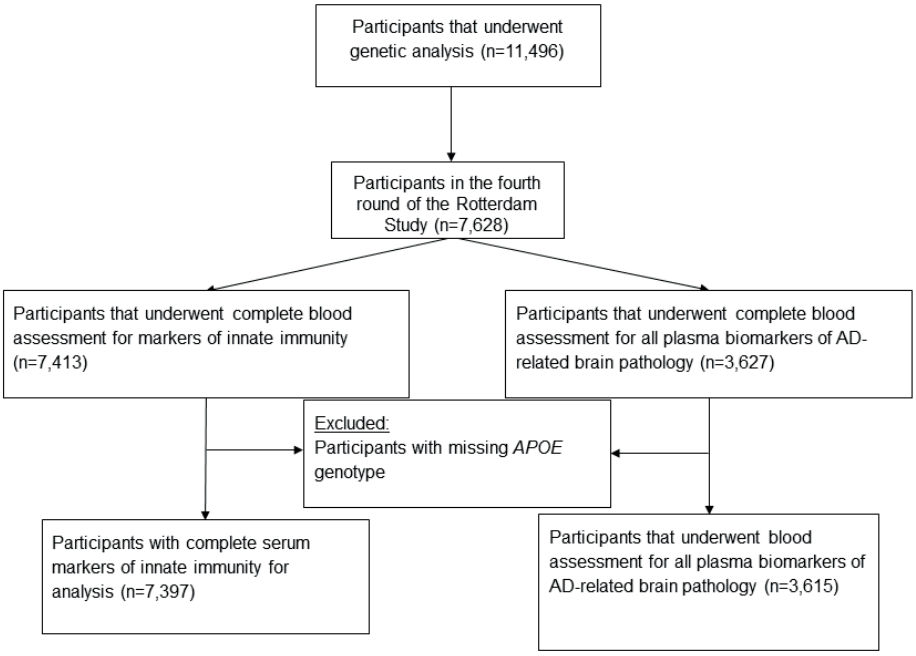


Figure 1. Flow Diagram

Assessment of blood cell counts and their derived ratios

Full blood count measurements were performed using the COULTER® Ac·T diff2™ Hematology Analyzer (Beckman Coulter, San Diego, California, USA) directly after blood sample drawn. Laboratory measurements included absolute granulocyte, platelet, and lymphocyte counts in 10^9 per litre. The GLR and PLR were calculated as the ratio of granulocyte count to lymphocyte count, and as the ratio of platelet count to lymphocyte count, respectively. The SII was defined as platelet count times the GLR [13]. We use the GLR as proxy measure for

the commonly used neutrophil-to-lymphocyte ratio, as granulocytes are the most abundant subtype of neutrophils.

Assessment of plasma Aβ, total-tau and NfL

Blood was sampled in EDTA treated containers and centrifuged. Subsequently plasma was aliquoted and frozen at -80^{0}C according to standard procedures. Measurements were done in two separate batches. The first batch included in total 2,000 samples, obtained from a random selection of 1,000 participants from sub-cohort RS-I-4 and 1,000 from RS-II-2. The second batch included in total 3,094 samples from the remaining participants.

All measurements were performed at Quanterix (Lexington, MA, USA) on a single molecule array (Simoa) HD-1 analyzer platform [23]. Samples were tested in duplicate. Two quality control (QC) samples were run on each plate for each analyte. NfL was measured with the NF-light advantage kit [24]. The Simoa Human Neurology 3-Plex A assay (N3PA) was used for measuring the concentration of total-tau, Aβ-42 and Aβ-40. When duplicates or single measurements were missing (the majority of missing samples were due to system failures (n=279), and few because of insufficient volume (n=47)) or in the case the concentration coefficient of variation (CV) exceeded 20% (14 to 87 samples) or control samples were out of range (none), participant's data were excluded from the analyses [16].

Genetic risk scores

We computed genetic risk scores, by selecting late-onset AD-associated single-nucleotide polymorphisms (SNPs) reaching genome-wide significance ($P < 5.0 \times 10^{-8}$). Among common variants, we considered only variants identified by the International Genomics of Alzheimer's Project (IGAP) meta-analyses [4]. In addition, we included four rare variants which can be classified under immune response based on their functional role [25, 26], leading to a total of 28 independent genome-wide significant AD-associated variants. A weighted genetic risk score was constructed using the effect sizes (log of odds ratio) of the genome-wide significant variants from the IGAP meta-analysis as weights and their respective allele dosages from imputed genotype data of our study cohorts. A genetic risk score was constructed as the sum of the products of SNP dosages and their corresponding weights. We constructed genetic risk scores in three ways: (1) combining all 28 selected variants, (2) excluding the *APOE*-ε4 variant to identify the joint independent effect of all other genome-wide significant SNPs and (3) clustering the variants into the immune response pathway. We classified AD associated SNPs into immune system pathway based on information on current investigations and reviews [4, 27-29]. Of the 28 SNPs, 9 could be clustered into the immune response pathway-based genetic risk score; **Table S1**. All genetic risk scores were standardized to allow direct comparison of results.

Covariates

Potential confounding factors were chosen on the basis of previous literature [13, 30]. All covariates were measured at the same rounds as the assessments of AD-related brain pathology and immunity markers. Smoking habits were categorized as current vs former and never smoking. Body mass index (BMI) was calculated as weight in kilograms per height in meters squared. Blood pressure was measured twice at the right brachial artery with the participant in sitting position, out of which the mean was used. Diabetes mellitus was defined as use of antidiabetic medication, fasting serum glucose level ≥7.1 mmol/L (≥127.9 mg/dL), or random non-fasting serum glucose level ≥11.1 mmol/L (≥200.0 mg/dL) [31]. Additional information from the serum samples was collected on high sensitivity C-reactive protein (hs-CRP) and creatinine in a subsample (N=1,342).

Statistical analysis

As the GLR, PLR and SII had a skewed distribution, analysis was based on the natural logarithmic (Ln) transformation of their values. For the same reason, all plasma biomarkers of AD-related brain pathology, except Aβ-40 which had a normal distribution, were Ln transformed. We first determined the association between the GLR, PLR and SII with plasma biomarkers of AD-related brain pathology using linear regression. For this analysis, we conducted two different transformations: 1) Because we transformed both exposure and outcome: $\log Yi = \alpha + \beta \log Xi + \varepsilon$, we reported mean differences in percentages with 95% confidence intervals (CIs) obtained by exponentiating Ln(2) times the estimated coefficients from the linear regression model to facilitate interpretation of results: These mean differences correspond to the percentage change in plasma Aβ, total-tau or NfL for a doubling of the GLR, PLR and SII. For this analysis, Aβ-40 was also Ln transformed. 2) We standardized all blood markers to allow direct comparison of the results. We adjusted all models for age, sex and study cohort (model I). In addition, the following covariates were added to a second model (model II):, smoking, diabetes, BMI and systolic blood pressure and *APOE*-ε4 carriership. Since plasma Aβ, total-tau and NfL were measured in two batches, we additionally corrected for batch effects in models. We performed additional adjustment for platelet count when analysing GLR in model II. We assessed effect measure modification by *APOE* genotype by stratifying for participants having the ε2/ε2 or ε2/ε3, ε3/ε3 and ε3/ε4 or ε4/ε4 genotype. We formally tested interaction between *APOE* genotype and ratios of blood cell counts on the multiplicative scale by adding interaction terms to model II. In addition, we assessed effect measure modification by activity of the innate immune system by stratifying for median granulocyte count (as pure innate immunity marker rather than studying the effect of the balance between innate and adaptive immunity) when assessing the associations between genetic risk scores and plasma biomarkers of AD-related brain pathology as outcome. We formally tested interaction between genetic risk scores and granulocyte counts on the multiplicative scale by adding interaction terms to the model. As a sensitivity analysis,

we also adjusted for creatinine and hs-CRP in a subsample of participants in which these markers were measured. Because hs-CRP is also an important inflammatory biomarker, we also assessed the association between Ln(hs-CRP) as exposure and the standardized plasma biomarkers of AD-related brain pathology as outcome using linear regression. We adjusted all models for age, sex, cohort, smoking, diabetes, BMI, systolic blood pressure, *APOE*-ε4 carriership, batch effects, platelet count and creatinine.

We then determined associations between all three genetic risk scores per standard deviation (SD) increase as exposure with the standardized immunity markers (Ln(GLR), Ln(PLR) and Ln(SII)) and with the standardized plasma biomarkers of AD-related brain pathology (Ln(Aβ-42, Aβ-40, Ln(Aβ-42/40 ratio), Ln(total-tau) and Ln(NfL)) as separate outcomes, using linear regression. These analyses were all adjusted for age, sex and study cohort.

Furthermore, we investigated the association between *APOE*-allele carriership as exposure with serum markers of immunity and plasma biomarkers of AD-related brain pathology as outcomes. For this analysis, we defined *APOE*-allele carriership as either carrying ε2 (ε2/ε2 or ε2/ε3 genotype), being homozygous for ε3 (ε3/ε3 genotype) or carrying ε4 (ε3/ε4 or ε4/ε4 genotype). People with the ε2/ε4 genotype were excluded from the analyses and ε3 homozygosity was considered the reference group. Analyses were adjusted for age, sex and study cohort. We calculated mean levels of hs-CRP and creatinine levels within the different *APOE* genotypes.

Missing covariate data (maximum 0.7%) were imputed using 5-fold Multiple Imputation by Chained Equations based on determinant, outcome, and included covariates. All analyses were performed using RStudio version 1.0.153 (R version 3.6.1, RStudio, Inc., Boston, MA). We corrected for multiple testing using Bonferroni adjustment for all analyses using the total population (i.e. not stratified or no subgroup analyses): For the associations between serum markers of immunity and plasma biomarkers of AD-related brain pathology, results are considered statistically significant if *P* below 0.05/(6×5)=0.002; for the associations of genetic risk scores reflecting AD including and excluding *APOE*-ε4 and immune response with plasma biomarkers of AD-related brain pathology and serum markers of immunity, results were considered statistically significant if *P* below 0.05/(9×5)=0.001 and for the associations of *APOE* genotypes with plasma biomarkers of AD-related brain pathology and serum markers of immunity, results are considered statistically significant if *P* below 0.05/16=0.003. For these analyses, a suggestive association was considered at the level alpha=0.05. All other analyses were considered statistically significant at the level alpha=0.05.

RESULTS

Characteristics of study participants are displayed in **Table 1**. The mean age of participants with serum markers of innate immunity was 66.1 (±10.4) years of which 4,208 (57%) were

Table 1. Characteristics of study population.

Characteristic	Sample with serum markers of immunity (N = 7,397)	Sample with plasma biomarkers of AD-related brain pathology (N = 3,615)
Women	4,208 (56.9%)	2,059 (56.8%)
Age, years	66.1 ±10.4	71.9 ±7.3
Study cohort		
Cohort 1	2,656 (35.9%)	2,159 (59.7%)
Cohort 2	1,704 (23.0%)	1,456 (40.3%)
Cohort 3	3,037 (41.1%)	-
Current smokers	1,427 (19.4%)	536 (14.9%)
Diabetes mellitus type 2	433 (5.9%)	214 (5.9%)
Body mass index, kg/m^2	27.6 ±4.3	27.6 ±4.1
Systolic blood pressure, mmHg	142.8 ±21.9	149.2 ±21.0
APOE genotype		
ε2/ε2	46 (0.6%)	31 (0.9%)
ε2/ε3	952 (12.9%)	490 (13.6%)
ε2/ε4	202 (2.7%)	103 (2.8%)
ε3/ε3	4293 (58.0%)	2119 (58.6%)
ε3/ε4	1741 (23.5%)	810 (22.4%)
ε4/ε4	163 (2.2%)	62 (1.7%)
Granulocyte count, × 10^3/microL	4.0 ±1.4	4.0 ±1.3
Platelet count, × 10^3/microL	268.2 ±67.2	256.4 ±64.4
Lymphocyte count, × 10^3/microL	2.3 ±1.2	2.2 ±1.3
Granulocyte-to-lymphocyte ratio	1.9 ±0.9	2.0 ±0.9
Platelet-to-lymphocyte ratio	128.2 ±47.3	130.0 ±49.8
Systemic immune-inflammation index	517.3 ±280.1	522.0 ±290.9
Amyloid-beta 42, pg/mL	-	10.6 ±3.0
Amyloid-beta 40, pg/mL	-	265.6 ±54.6
Amyloid-beta 42/40 ratio	-	0.04 ±0.009
Total-tau, pg/mL	-	2.6 ±2.5
Neurofilament light chain, pg/mL	-	15.7 ±11.6

Abbreviations: N = number of participants included in study. Data presented as mean (standard deviation) for continuous variables and number (percentages) for categorical variables. Data here are unimputed. Number of missing values for the immunity cohort: 39 (0.5%) for smoking, 19 (0.3%) for diabetes, 20 (0.3%) for BMI, 37 (0.5%) for systolic blood pressure, 0 for *APOE* carriership. Number of missing values for the sample undergoing serum AD measurements are: 25 (0.7%) for smoking, 8 (0.2%) for diabetes, 0 for BMI, 12 (0.3%) for systolic blood pressure and 0 for *APOE* genotype.

women and the mean age of the sample undergoing serum AD marker measurements was 71.9 (±7.3) years of which 2,059 (57%) were women.

We found that a doubling of SII was associated with a 1.2% higher serum Aβ-42, albeit not statistically significant (95% confidence interval (95% CI) -0.005% to 2.3%, *P*=0.051) and a 1.8% higher serum Aβ-40 (95% CI 0.9 to 2.7%, *P*=<0.001). Estimates were even higher for a doubling of GLR, leading to a 1.9% higher serum Aβ-42 (95% CI 0.4 to 3.3%, *P*=0.010) and 3.2% higher serum Aβ-40 (95% CI 2.0 to 4.3%, *P*=<0.001). The Aβ-42/40 ratio was lower with a doubling in GLR only (-1.2% [-2.3 to -0.1%], *P*=0.028). Only a doubling in PLR was significantly associated with lower total-tau (-3.6% [-5.5% to -1.7%], *P*=<0.001). NfL was higher with all innate immunity phenotypes, most strongly with a doubling in GLR (4.7% [2.5 to 7.1%], *P*=<0.001); **Table 2** and **Table S2**. Stratification did not reveal statistically significant differences across strata of *APOE* carriership, with the effects in the *APOE* ε3/ε4 or ε4/ε4 stratum being only non-significantly stronger than in the other strata, especially for NfL; **Figure 2** and **Table S2**. In addition, the associations between all genetic risk scores with lower Aβ-42/40 ratio became lower in participants with granulocyte counts higher than the median (mean difference = -0.18, 95% = CI -0.23; -0.13, *P*=<0.001 for the overall AD genetic risk score, mean difference = -0.06, 95% CI = -0.11; -0.02, *P*=0.007 for the overall AD genetic risk score excluding *APOE* and mean difference = -0.05, 95% CI = -0.10; -0.004, *P*=0.035 for the immune response pathway-based genetic risk score, yet the interaction remained non-significant; **Table S3**). When additionally adjusting for hs-CRP and creatinine (in a subsample: N=1,341), the associations between GLR and SII with higher Aβ-42 and NfL disappeared, the associations between immunity markers with higher Aβ-40 were borderline significant and the associations with lower Aβ-42/40 ratio and lower total-tau persisted; **Table S4**. Higher hs-CRP was significantly associated with a higher Aβ-40 and higher total-tau; **Table S5**.

Furthermore, we found that a standard deviation (SD) increase in the overall AD genetic risk score including *APOE*-ε4 was associated with higher GLR (mean difference in Ln(GLR) [SD] = 0.024, 95% CI = 0.002; 0.046, *P*=0.032) and significantly with lower Aβ-42 and Aβ-42/40 ratio (mean difference in Aβ-42 [SD] = -0.129, 95% CI = -0.162; -0.095, *P*=<0.001 and Ln(Aβ-42/40) [SD] = -0.157, 95% CI = -0.190; -0.124, *P*=<0.001, respectively). These associations were mainly driven by the *APOE*-ε4 variant; **Figure 3**. The genetic risk score reflecting the immune response showed a suggestive association with lower Aβ-42/40 ratio (mean difference = -0.038, 95% CI = -0.070; -0.006, *P*=0.020), but not with the serum markers of innate immunity; **Figure 3** and **Table S6**. We found that *APOE*-ε2 carriers displayed lower serum markers of innate immunity levels compared to ε3/ε3, while these markers were elevated in *APOE*-ε4; **Figure 4** and **Table S7**. Mean levels of hs-CRP and creatinine levels within the different *APOE* genotypes are shown in **Table S8**.D

Table 2. Associations between serum markers of immunity and plasma biomarkers of AD-related brain pathology.

Per doubling in serum markers of immunity	Percentage change in Aβ, total-tau or NFL, 95% CI									
	Aβ-42	P	Aβ-40	P	Aβ-42/40 ratio	P	Tau	P	NFL	P
Model I										
GLR*	1.6 (0.2; 3.1)	0.025	3.2 (2.1; 4.3)	<0.001	-1.5 (-2.6; -0.4)	0.008	0.1 (-1.8; 2.0)	<0.001	4.9 (2.5; 7.2)	<0.001
PLR	-0.7 (-2.2; 0.8)	0.351	-0.2 (-1.4; 0.9)	0.688	-0.5 (-1.6; 0.7)	<0.001	-4.4 (-6.3; -2.5)	<0.001	2.9 (0.5; 5.3)	0.018
SII	1.0 (-0.1; 2.2)	0.078	1.8 (0.9; 2.7)	<0.001	-0.8 (-1.7; 0.1)	0.093	-0.4 (-1.9; 1.1)	0.590	3.3 (1.5; 5.2)	<0.001
Model II										
GLR*	1.9 (0.4; 3.3)	0.010	3.2 (2.0; 4.3)	<0.001	-1.2 (-2.3; -0.1)	0.028	-0.1 (-1.9; 1.8)	0.927	4.7 (2.5; 7.1)	<0.001
PLR	-0.8 (-2.2; 0.7)	0.314	-0.2 (-1.3; 1.0)	0.790	-0.6 (-1.8; 0.6)	0.309	-3.6 (-5.5; -1.7)	<0.001	1.5 (-0.8; 3.9)	0.205
SII	1.2 (0.00; 2.3)	0.051	1.8 (0.9; 2.7)	<0.001	-0.6 (-1.5; 0.3)	0.190	-0.5 (-2.0; 1.0)	0.539	2.9 (1.1; 4.8)	0.002

Abbreviations: CI = confidence interval, Ln = natural logarithmic transformation, GLR = granulocyte-to-lymphocyte ratio, PLR = platelet-to-lymphocyte ratio, SII = systemic immune-inflammation index, Aβ-42 = amyloid-beta 42, Aβ-40 = amyloid-beta 40, Aβ-42/40 ratio = amyloid-beta 42-to-40 ratio, Tau = total-tau, NFL = neurofilament light chain. Model I adjusted for age, sex, study cohort and batch effects. Model II adjusted for age, sex, study cohort, smoking, diabetes, BMI, platelets, systolic blood pressure, *APOE*-ε4 and batch effects. The GLR, PLR and SII reflect the balance between innate and adaptive immunity, with higher markers indicating a dysbalance towards higher innate immunity. *Additional adjustment for platelet count. Results are considered statistically significant if *P* below 0.05/(6×5)=0.002.DSupporting information

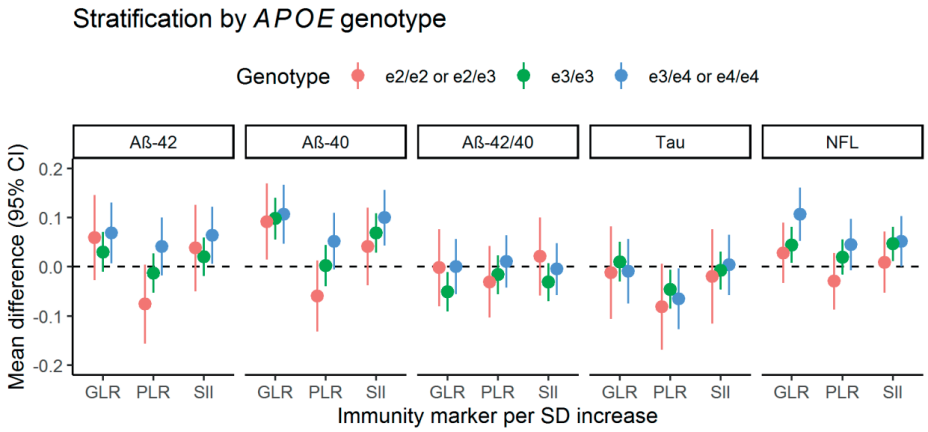


Figure 2. Associations between serum markers of immunity and plasma biomarkers of AD-related brain pathology.

Abbreviations: CI = confidence interval, SD = standard deviation, Ln = natural logarithmic transformation, GLR = granulocyte-to-lymphocyte ratio, PLR = platelet-to-lymphocyte ratio, SII = systemic immune-inflammation index, Aβ-42 = amyloid-beta 42, Aβ-40 = amyloid-beta 40, Aβ-42/40 ratio = amyloid-beta 42-to-40 ratio, NFL = neurofilament light chain, e2/e2 or e2/e3 = apolipoprotein Eε2/ε2 genotype, e3/e3 = apolipoprotein Eε3/ε3 genotype, e3/e4 or e4/e4 = apolipoprotein Eε3/ε4 or ε4/ε4 genotype. Model adjusted for age, sex, study cohort, smoking, diabetes, BMI, platelets, systolic blood pressure, *APOE*-ε4 and batch effects. Additional adjustment for platelet count when analysing Ln(GLR). All phenotypical markers were Ln(transformed) except for Aβ-40. The GLR, PLR and SII reflect the balance between innate and adaptive immunity, with higher markers indicating a dysbalance towards higher innate immunity.

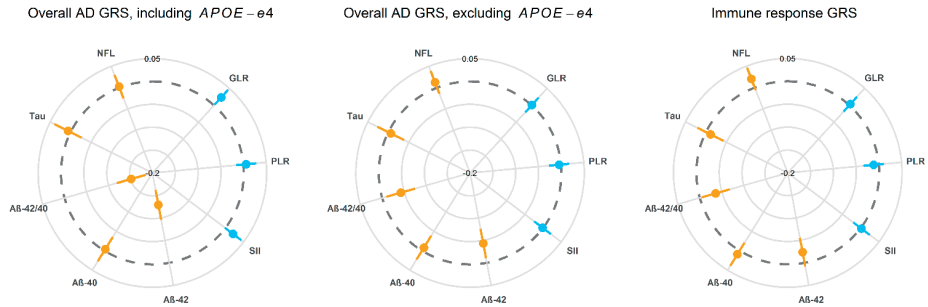


Figure 3. Associations of genetic risk scores reflecting AD including and excluding *APOE*-ε4 and immune response with plasma biomarkers of AD-related brain pathology and serum markers of immunity.

Abbreviations: Ln = natural logarithmic transformation, AD = Alzheimer's disease, GRS = genetic risk score, GLR = granulocyte-to-lymphocyte ratio, PLR = platelet-to-lymphocyte ratio, SII = systemic immune-inflammation index, Aβ-42 = Amyloid-beta 42, Aβ-40 = Amyloid-beta 40, Aβ-42/40 = Amyloid-beta 42-to-40 ratio, NFL = neurofilament light chain. Model adjusted for age, sex and

study cohort. GRS are per SD increase. All phenotypical markers were Ln(transformed) except for Aβ-40. Gray dotted circle indicates the null with associations becoming increasingly negative towards the centre of the circle. Orange indicates the plasma biomarkers of AD-related brain pathology while blue indicates the immunity phenotypes. The GLR, PLR and SII reflect the balance between innate and adaptive immunity, with higher markers indicating a dysbalance towards higher innate immunity.

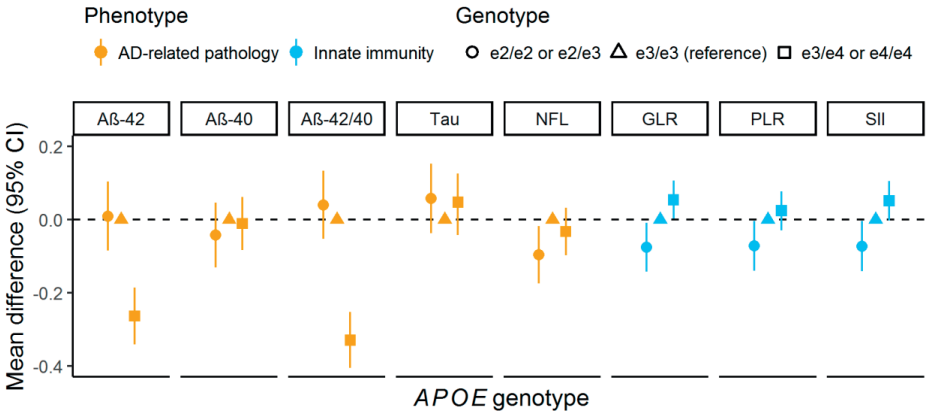


Figure 4. Associations of *APOE* genotypes with plasma biomarkers of AD-related brain pathology and serum markers of immunity.

Abbreviations: CI = confidence interval, SD = standard deviation, GRS = genetic risk score, AD = Alzheimer's disease, Ln = natural log transformation, GLR = granulocyte-to-lymphocyte ratio, SII = systemic immune inflammation index, Aβ-42 = Amyloid-beta 42, Aβ-40 = Amyloid-beta 40, Aβ-42/40 = Amyloid-beta 42-to-40 ratio, NFL = neurofilament light chain, *APOE* = Apolipoprotein E, e2/e2 or e2/e3 = apolipoprotein Eε2/ε2 genotype, e3/e3 = apolipoprotein Eε3/ε3 genotype, e3/e4 or e4/e4 = apolipoprotein Eε3/ε4 or ε4/ε4 genotype. Adjusted for age, sex and study cohort. All phenotypical markers were Ln(transformed) except for Aβ-40. The GLR, PLR and SII reflect the balance between innate and adaptive immunity, with higher markers indicating a dysbalance towards higher innate immunity.

DISCUSSION

In this study, we found associations between higher levels of GLR, PLR and SII reflecting higher innate immunity, with higher Aβ42 and 40, lower Aβ-42/40 ratio, lower total-tau and higher NfL in plasma. In *APOE*-ε4 carriers, these associations were even stronger. The overall genetic risk score including *APOE*-ε4 was associated with higher GLR and SII and with lower Aβ-42 and Aβ-42/40 ratio. These effects were mainly driven by the *APOE*-ε4 variant. The genetic risk score reflecting the immune response was associated with lower Aβ-42/40 ratio, but not with the serum immunity markers. Furthermore, we found that *APOE*-ε2 carriers displayed lower serum markers of innate immunity compared to *APOE*-ε3/ε3, while these markers were elevated in *APOE*-ε4 carriers.

Interestingly, we found that higher innate immunity was associated with higher serum Aβ-42, but with even higher Aβ-40. The 40-residue peptide represents the most abundant Aβ isoform in the brain [32], while the 42-residue shows a significant increase with certain forms of AD. Our findings are concordant with the notion that Aβ functions as an anti-microbial peptide (AMP), because the physiochemical and biological properties previously reported for Aβ are similar to those of AMPs. In addition, experiments have shown that Aβ is active against at least eight common and clinically relevant microorganisms [33]. Activity was isoform-specific for six organisms with Aβ-42 showing greater potency compared to Aβ40 [33]. Taken all evidence together, we propose that *APOE-ε4* carriers display stronger innate immune responses, and thus produce more/excess Aβ in response to pathogens. Of the two isoforms, Aβ42 will aggregate in the brain (possibly due to its larger size) while Aβ40 will not aggregate (or to a lesser extent) and as a consequence will be higher in serum. Further study is needed to confirm this hypothesis.

Furthermore, we found that both higher serum markers of innate immunity and the genetic risk score reflecting the immune response associate with the lower serum Aβ-42/40 ratio. *APOE*-ε4 carriership, which is the major genetic risk factor for AD, also displays lower serum Aβ-42/40 ratio as well as lower Aβ-42. In line with this, a meta-analysis of prospective cohort studies has shown that lower serum Aβ-40, even lower Aβ-42 and consequently lower Aβ-42/40 ratio lead to higher AD risk.[34] Previous reports of associations of low plasma Aβ-42/40 ratio with increased amyloid brain uptake, as measured by Pittsburgh compound B (PiB) positron emission tomography (PET) scan, support the notion that lower plasma Aβ reflect the aggregation of Aβ in the brain.[35-38] In this context, our finding that *APOE*-ε4 carriers have higher activity of innate immunity compared to ε3/ε3, while we see the opposite in *APOE*-ε2 carriers suggests that *APOE*-ε4 might have an overactive innate immune response. Our results support experimental studies showing the capacity of *APOE* to modulate inflammation. Indeed, in healthy humans challenged with intravenous lipopolysacharide (LPS) infusion, ε4 carriers demonstrated significantly higher elevation of body temperature and plasma tumor necrosis factor levels than ε4 non-carriers. [39] In this same study, when whole blood isolates from human subjects were stimulated ex-*vivo* with Toll-like receptor ligands, increased production of a wide panel of cytokines and chemokines was observed in blood from ε4+ donors compared with ε4– donors. A higher immune response associated with the ε4 allele is also observed in human *APOE*-targeted replacement mice and in cultured microglia and/or macrophages upon LPS stimulation.[39-41] How *APOE* achieves this is not yet well-understood.[8] Evidence shows that lipid rafts play an essential role in immune activation by serving as platforms for signaling complexes. [42] *APOE*-ε4 is reported to be less effective than *APOE*-ε3 in inducing cholesterol efflux from macrophages[43], which leads to cholesterol accumulation on cell membranes.[39, 43] This mechanism has been proposed to explain the higher immune reactivity associated with

APOE-ε4[8], but further studies are needed to explore this or potential other mechanisms further.

In addition to the role of immune response pathways in Aβ, our data show that higher innate immunity related to lower plasma total-tau. Others assessed the effect of microglial activation on tau pathogenesis, where they found that microglial activation is shown to precede tau pathology in a tauopathy mouse model (Yoshiyama et al. 2007) and administering an immunosuppressant drug FK506 from an early age drastically reduces tau pathology [44]. According to previous studies, higher plasma tau is associated with AD dementia [16, 45], although correlations were weak [45] and non-linear (J-shape) [16], making the role of immunity in tau elusive. Future studies are implicated relating the same crude markers of the immune system in relation to phospho tau.

The associations between immunity markers with higher Aβ-42 and NfL disappeared when additionally adjusting for hs-CRP and creatinine. There could be two explanations: 1) This analysis was performed within a subsample of the total population, impeding comparison of the results in the total population and decreasing the power of the analysis. 2) We found that higher hs-CRP was also associated with higher NfL, albeit not statistically significant, suggesting that hs-CRP could be acting as a mediator.

Our result that the immune GRS was not related to the immunity biomarker levels is surprising, especially since several immune genes are key players for innate immunity, including *TREM2* [46]. TREM2 overexpression is thought to enhance microglial phagocytotic capacity, but transcription analyses show mixed microglial activation patterns with suppression of certain disease-associated microglia (DAM) genes, but further activation patterns of other DAM genes [47], making the role of *TREM2* in AD unclear. Further studies are needed to identify the precise gene signatures of microglia that mediate pathology-and neurodegeneration-associated sterile inflammation.

Our study has several limitations. First, ours is a cross-sectional study, limiting our ability to draw causal inferences. Longitudinal cohort studies are required to confirm our findings. Second, we were limited to studying crude markers of the immune system in this population-based setting. These types of measures have been associated with other outcomes like cancer-related, pancreatitis, and many other responses [48]. These ratios could be proxies for different systemic inflammatory responses that affect mobilization of bone marrow-derived cells and egress of leukocytes into tissue [49]. It could also be related to immunosenescence and this might explain the association with NfL because it too, increases with age [50]. Third, we were similarly limited to crude markers of AD-related brain pathology which are not diagnostic of AD, especially since the ATN-classification includes AD biomarkers as measured by PET, structural MRI or CSF and we do not have phospho tau measured. However, plasma and CSF levels of these biomarkers are strongly correlated [51-53]. Also NfL is a global biomarker and not specific for AD. Fourth, we were unable to categorise the age groups into early and late onset AD respectively, due to the low number of early

onset AD patients within our population-based cohort. It would be helpful to categorise the biomarkers according to early and late onset AD in future studies, because Aβ has a better correlation in early onset AD. Early onset AD differs from late onset AD not only with respect to genetic predisposition and pathology but also in relation to the clinical outcome and the natural course. Only 11% of early AD patients has familial mutations with *APP*, *PSEN1* and *PSEN2*. For example, patients with early AD have a greater burden in precuneus and parietal lobes and to a lesser extent in the frontal lobes [54]. Fifth, we did not offer replication data. We hope that our study provides a stimulus for other cohort studies to replicate our findings. Lastly, our study contains predominantly Caucasians, which limits its external generalizability. Further study in other ethnicities is needed to identify potential ethnic differences.

In summary, in this population-based study we showed that higher innate immunity, as reflected by higher levels of serum GLR, PLR and SII, were associated with higher plasma Aβ42, 40, and NfL, and with lower Aβ-42/40 and total-tau. Furthermore, *APOE*-ε2 carriers displayed lower serum markers of innate immunity levels compared to ε3/ε3, while these markers were elevated in *APOE*-ε4. Knowledge on the potential pro-inflammatory role of *APOE*-ε4 should encourage future studies to find ways to scale down innate immune responses in *APOE*-ε4 carriers to limit AD-related brain pathology to prevent AD.

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SUPPORTING INFORMATION

Table S1. Clustering of genome-wide significant variants into the immune response pathway.

Pathway	Gene	Rare or common	Assigned SNP	Kunkle et al. [4]	Dourlen et al. [22]	Jones et al. [23]	Guerreiro et al. [24]
Immune Response	CLU	Common	rs9331896	Yes	Yes	Yes	Yes
	CR1	Common	rs4844610	Yes	Yes	Yes	Yes
	INPP5D	Common	rs10933431	Yes	Yes	Yes	Yes
	EPHA1	Common	rs10808026	-	-	-	Yes
	MS4A2	Common	rs7933202	-	Yes	-	Yes
	MEF2C	Common	rs190982	Yes	-	-	Yes
	ABI3	Rare	rs616338	-	Yes	-	-
	PLCG2	Rare	rs72824905	Yes	Yes	Yes	-
	TREM2	Rare	rs75932628	-	Yes	-	Yes
	TREM2	Rare	rs143332484	-	Yes	-	-

Table S2. Associations between serum markers of immunity and plasma biomarkers of AD-related brain pathology stratified for *APOE* genotype.

Per SD increase in serum markers of immunity	SD change in Aβ, total-tau or NFL, 95% CI									
	Ln(Aβ-42)	P	Aβ-40	P	Ln(Aβ-42/40)	P	Ln(Tau)	P	Ln(NFL)	P
APOE ε2/ε2 or ε2/ε3										
Ln(GLR)*	0.06 (-0.03; 0.15)	0.177	0.09 (0.01; 0.17)	0.021	0.00 (-0.08; 0.08)	0.964	-0.01 (-0.11; 0.08)	0.801	0.03 (-0.03; 0.09)	0.367
Ln(PLR)	-0.08 (-0.16; 0.00)	0.064	-0.06 (-0.13; 0.01)	0.106	-0.03 (-0.10; 0.04)	0.406	-0.08 (-0.17; 0.01)	0.069	-0.03 (-0.09; 0.03)	0.312
Ln(SII)	0.04 (-0.05; 0.13)	0.394	0.04 (-0.04; 0.12)	0.307	0.02 (-0.06; 0.10)	0.607	-0.02 (-0.12; 0.08)	0.687	0.01 (-0.05; 0.07)	0.773
APOE ε3/ε3										
Ln(GLR)*	0.03 (-0.01; 0.07)	0.147	0.10 (0.06; 0.14)	<0.001	-0.05 (-0.09; -0.01)	0.013	0.01 (-0.03; 0.05)	0.627	0.04 (0.01; 0.08)	0.018
Ln(PLR)	-0.01 (-0.05; 0.03)	0.527	0.00 (-0.04; 0.04)	0.911	-0.02 (-0.06; 0.02)	0.428	-0.05 (-0.09; -0.01)	0.024	0.02 (-0.02; 0.06)	0.283
Ln(SII)	0.02 (-0.02; 0.06)	0.301	0.07 (0.03; 0.11)	0.001	-0.03 (-0.07; 0.01)	0.109	-0.01 (-0.05; 0.03)	0.695	0.05 (0.01; 0.08)	0.008
APOE ε3/ε4 or ε4/ε4										
Ln(GLR)*	0.07 (0.01; 0.13)	0.030	0.11 (0.05; 0.17)	0.001	0.00 (-0.06; 0.06)	0.994	-0.01 (-0.07; 0.06)	0.781	0.11 (0.05; 0.16)	<0.001
Ln(PLR)	0.04 (-0.02; 0.10)	0.174	0.05 (-0.01; 0.11)	0.078	0.01 (-0.04; 0.06)	0.697	-0.07 (-0.13; 0.003)	0.040	0.04 (-0.01; 0.10)	0.094
Ln(SII)	0.06 (0.01; 0.12)	0.031	0.10 (0.04; 0.16)	0.001	0.00 (-0.06; 0.05)	0.859	0.004 (-0.06; 0.07)	0.901	0.05 (0.00001; 0.10	0.050
APOE genotype	**P for interaction**									
Ln(GLR)*	0.364		0.994		0.198		0.298		0.169	
Ln(PLR)	0.696		0.418		0.990		0.801		0.515	
Ln(SII)	0.518		0.624		0.690		0.930		0.931	

Abbreviations: SD = standard deviation, CI = confidence interval, Ln = natural logarithmic transformation, GLR = granulocyte-to-lymphocyte ratio, PLR = platelet-to-lymphocyte ratio, SII = systemic immune-inflammation index, Aβ-42 = amyloid-beta 42, Aβ-40 = amyloid-beta 40, Aβ-42/40 ratio = amyloid-beta 42-to-40 ratio, Tau = total-tau, NFL = neurofilament light chain. Model adjusted for age, sex, study cohort, smoking, diabetes, BMI, platelets, systolic blood pressure and batch effects. The GLR, PLR and SII reflect the balance between innate and adaptive immunity, with higher markers indicating a dysbalance towards higher innate immunity. *Additional adjustment for platelet count.

Table S3. Associations of genetic risk scores reflecting AD including and excluding *APOE*-ε4 and immune response with plasma biomarkers of AD-related brain pathology and serum markers of immunity stratified for median granulocyte counts.

Per SD increase in genetic risk scores	SD change in Aβ, total-tau or NFL, 95% CI									
	Ln(Aβ-42)	P	Aβ-40	P	Ln(Aβ-42/40)	P	Ln(Tau)	P	Ln(NFL)	P
Granulocyte counts lower than the median										
Overall AD GRS, including APOE-ε4	-0.10 (-0.15; -0.06)	<0.001	0.00 (-0.04; 0.04)	0.942	-0.13 (-0.18; -0.09)	<0.001	0.00 (-0.05; 0.05)	0.972	0.02 (-0.02; 0.06)	0.285
Overall AD GRS, excluding APOE-ε4	-0.04 (-0.09; 0.00)	0.057	-0.03 (-0.07; 0.01)	0.204	-0.03 (-0.07; 0.02)	0.237	-0.01 (-0.06; 0.04)	0.682	0.00 (-0.04; 0.04)	0.886
Immune response GRS	-0.03 (-0.08; 0.01)	0.129	-0.01 (-0.05; 0.03)	0.672	-0.03 (-0.07; 0.01)	0.156	-0.04 (-0.09; 0.00)	0.079	0.02 (-0.02; 0.06)	0.308
Granulocyte counts higher than the median										
Overall AD GRS, including APOE-ε4	-0.15 (-0.20; -0.11)	<0.001	-0.02 (-0.06; 0.03)	0.523	-0.18 (-0.23; -0.13)	<0.001	0.01 (-0.04; 0.06)	0.692	-0.02 (-0.06; 0.02)	0.321
Overall AD GRS, excluding APOE-ε4	-0.05 (-0.10; 0.00)	0.047	0.01 (-0.04; 0.05)	0.773	-0.06 (-0.11; -0.02)	0.007	0.00 (-0.05; 0.04)	0.958	0.02 (-0.02; 0.06)	0.369
Immune response GRS	-0.02 (-0.07; 0.02)	0.318	0.02 (-0.02; 0.07)	0.344	-0.05 (-0.10; -0.00)	0.035	0.01 (-0.03; 0.06)	0.585	0.02 (-0.02; 0.06)	0.312
	P for interaction									
Overall AD GRS, including APOE-ε4	0.996		0.512		0.292		0.526		0.387	
Overall AD GRS, excluding APOE-ε4	0.591		0.654		0.512		0.488		0.387	
Immune response GRS	0.794		0.953		0.657		0.640		0.615	

Abbreviations: SD = standard deviation, Ln = natural logarithmic transformation, AD = Alzheimer's disease, GRS = genetic risk score, GLR = granulocyte-to-lymphocyte ratio, PLR = platelet-to-lymphocyte ratio, SII = systemic immune-inflammation index, Aβ-42 = Amyloid-beta 42, Aβ-40 = Amyloid-beta 40, Aβ-42/40 = Amyloid-beta 42-to-40 ratio, NFL = neurofilament light chain. Model adjusted for age, sex and study cohort. GRS are per SD increase. All phenotypical markers were Ln(transformed) except for Aβ-40. The GLR, PLR and SII reflect the balance between innate and adaptive immunity, with higher markers indicating a dysbalance towards higher innate immunity.

Table S4. Associations between serum markers of immunity and plasma biomarkers of AD-related brain pathology with additional adjustments.

	SD increase in Aβ, total-tau or NFL, 95% CI									
Adjustments for creatinine and hs-CRP, N=1341	**Ln(Aβ-42)**	**P**	**Aβ-40**	**P**	**Ln(Aβ-42/40)**	**P**	**Ln(Tau)**	**P**	**Ln(NFL)**	**P**
Ln(GLR)*	0.003 (-0.049; 0.055)	0.922	0.045 (-0.001; 0.091)	0.054	-0.039 (-0.096; 0.019)	0.187	-0.055 (-0.106; -0.004)	0.036	-0.031 (-0.073; 0.010)	0.135
Ln(PLR)	-0.033 (-0.080; 0.014)	0.166	0.017 (-0.024; 0.058)	0.415	-0.056 (-0.106; -0.003)	0.038	-0.063 (-0.109; -0.017)	0.007	-0.010 (-0.048; 0.027)	0.583
Ln(SII)	0.002 (-0.046; 0.051)	0.923	0.040 (-0.003; 0.083)	0.070	-0.030 (-0.084; 0.024)	0.275	-0.050 (-0.099; -0.002)	0.041	-0.018 (-0.056; 0.021)	0.371

Abbreviations: SD= standard deviation, hs-CRP = high-sensitive CRP, CI = confidence interval, Ln = natural log transformation, GLR = granulocyte-to-lymphocyte ratio, SII = systemic immune inflammation index, Aß-42 = Amyloid-beta 42, Aß-40 = Amyloid-beta 40, Aß-42/40 = Amyloid-beta 42-to-40 ratio, NFL = neurofilament light chain. Model II: Adjusted for smoking, diabetes, BMI, platelets, systolic blood pressure, apolipoprotein E4ε carriership and batch effects. *Additional adjustment for platelet count.

Table S5. Associations between serum markers of immunity and plasma biomarkers of AD-related brain pathology.

Per log increase of hs-CRP N= 1341	SD increase in Aβ, total-tau or NFL, 95% CI									
	Ln(Aβ-42)	**P**	**Aβ-40**	**P**	**Ln(Aβ-42/40)**	**P**	**Ln(Tau)**	**P**	**Ln(NFL)**	**P**
Ln(CRP)	0.02 (-0.03; 0.06)	0.481	0.06 (0.02; 0.11)	0.002	-0.03 (-0.08; 0.02)	0.226	0.07 (0.03; 0.12)	0.002	0.03 (-0.01; 0.07)	0.124

Abbreviations: SD = standard deviation, hs-CRP = high-sensitive CRP, CI = confidence interval, Ln = natural logarithmic transformation, GLR = granulocyte-to-lymphocyte ratio, PLR = platelet-to-lymphocyte ratio, SII = systemic immune-inflammation index, Aβ-42 = amyloid-beta 42, Aβ-40 = amyloid-beta 40, Aβ-42/40 ratio = amyloid-beta 42-to-40 ratio, Tau = total-tau, NFL = neurofilament light chain. Model adjusted for age, sex, study cohort, smoking, diabetes, BMI, platelets, systolic blood pressure, *APOE*-ε4, batch effects and creatinine.D

Table S6. Associations of genetic risk scores reflecting AD including and excluding *APOE*-ε4 and immune response with plasma biomarkers of AD-related brain pathology and serum markers of immunity.

Per SD increase in genetic risk scores	SD change in Aβ, total-tau or NFL, 95% CI									
	Ln(Aβ-42)	P	Aβ-40	P	Ln(Aβ-42/40)	P	Ln(Tau)	P	Ln(NFL)	P
Overall AD GRS, including APOE-ε4	-0.13 (-0.16; -0.10)	<0.001	0.00 (-0.04; 0.03)	0.779	-0.16 (-0.19; -0.12)	<0.001	0.01 (-0.03; 0.04)	0.742	0.00 (-0.03; 0.03)	0.973
Overall AD GRS, excluding APOE-ε4	-0.04 (-0.08; -0.01)	0.007	-0.01 (-0.04; 0.02)	0.591	-0.05 (-0.08; -0.01)	0.006	-0.01 (-0.04; 0.03)	0.670	0.01 (-0.02; 0.04)	0.424
Immune response GRS	-0.02 (-0.06; 0.01)	0.138	0.01 (-0.02; 0.04)	0.542	-0.04 (-0.07; -0.01)	0.020	-0.01 (-0.04; 0.02)	0.456	0.02 (-0.01; 0.05)	0.151
Per SD increase in genetic risk scores	**SD change in GLR, PLR and SII**									
	Ln(GLR)	**P**	**Ln(PLR)**	**P**	**Ln(SII)**	**P**				
Overall AD GRS, including APOE-ε4	0.02 (0.00; 0.05)	0.032	0.01 (-0.02; 0.03)	0.579	0.02 (0.00; 0.04)	0.062				
Overall AD GRS, excluding APOE-ε4	0.00 (-0.02; 0.02)	0.921	0.00 (-0.03; 0.02)	0.715	0.00 (-0.02; 0.02)	0.936				
Immune response GRS	0.00 (-0.02; 0.03)	0.760	-0.01 (-0.03; 0.01)	0.343	0.00 (-0.02; 0.03)	0.833				

Abbreviations: SD = standard deviation, Ln = natural logarithmic transformation, AD = Alzheimer's disease, GRS = genetic risk score, GLR = granulocyte-to-lymphocyte ratio, PLR = platelet-to-lymphocyte ratio, SII = systemic immune-inflammation index, Aβ-42 = Amyloid-beta 42, Aβ-40 = Amyloid-beta 40, Aβ-42/40 = Amyloid-beta 42-to-40 ratio, NFL = neurofilament light chain. Model adjusted for age, sex and study cohort. GRS are per SD increase. All phenotypical markers were Ln(transformed) except for Aβ-40. The GLR, PLR and SII reflect the balance between innate and adaptive immunity, with higher markers indicating a dysbalance towards higher innate immunity. Results are considered statistically significant if *P* below 0.05/(9×5)=0.001.

Table S7. Associations of *APOE* genotypes with plasma biomarkers of AD-related brain pathology and serum markers of immunity.

APOE genotypes	SD change in Aβ, total-tau or NFL, 95% CI									
	Ln(Aβ-42)	**P**	**Aβ-40**	**P**	**Ln(Aβ-42/40)**	**P**	**Ln(Tau)**	**P**	**Ln(NFL)**	**P**
APOE ε2/ε2 or ε2/ε3	0.01 (-0.08; 0.10)	0.845	-0.04 (-0.13; 0.05)	0.343	0.04 (-0.05; 0.13)	0.402	0.06 (-0.04; 0.15)	0.231	-0.10 (-0.17; -0.02)	0.016
APOE ε3/ε3 (reference)	0	-	0	-	0	-	0	-	0	-
APOE ε3/ε4 or ε4/ε4	-0.26 (-0.34; -0.19)	<0.001	-0.01 (-0.08; 0.06)	0.766	-0.33 (-0.41; -0.25)	<0.001	0.05 (-0.03; 0.12)	0.237	-0.03 (-0.10; 0.03)	0.315
APOE genotypes	**SD change in GLR, PLR and SII**									
	Ln(GLR)	**P**	**Ln**(PLR)	**P**	**Ln**(SII)	**P**				
APOE ε2/ε2 or ε2/ε3	-0.08 (-0.14; -0.01)	0.025	-0.07 (-0.14; 0.00)	0.037	-0.07 (-0.14; 0.00)	0.038				
APOE ε3/ε3 (reference)	0	-	0	-	0	-				
APOE ε3/ε4 or ε4/ε4	0.05 (0.00; 0.11)	0.041	0.02 (-0.03; 0.08)	0.379	0.05 (0.00; 0.10)	0.064				

Abbreviations: CI = confidence interval, SD = standard deviation, GRS = genetic risk score, AD = Alzheimer's disease, Ln = natural log transformation, GLR = granulocyte-to-lymphocyte ratio, SII = systemic immune inflammation index, Aβ-42 = Amyloid-beta 42, Aβ-40 = Amyloid-beta 40, Aβ-42/40 = Amyloid-beta 42-to-40 ratio, NFL = neurofilament light chain, *APOE* = Apolipoprotein E, e2/e2 or e2/e3 = apolipoprotein Eε2/ε2 genotype, e3/e3 = apolipoprotein Eε3/ε3 genotype, e3/e4 or e4/e4 = apolipoprotein Eε3/ε4 or ε4/ε4 genotype. Adjusted for age, sex and study cohort. All phenotypical markers were Ln(transformed) except for Aβ-40. The GLR, PLR and SII reflect the balance between innate and adaptive immunity, with higher markers indicating a dysbalance towards higher innate immunity. Results are considered statistically significant if *P* below 0.05/16=0.003.

Table S8. Descriptive statistics of high-sensitive CRP and creatinine levels within *APOE* genotypes.

APOE genotype	Mean hs-CRP level	Mean creatinine level
ε2/ε2 or ε2/ε3 (N=203)	3.5	82.1
ε3/ε3 (N=799)	3.4	84.3
ε3/ε4 or ε4/ε4 (N=304)	2.3	84.3

Abbreviations: hs-CRP = high-sensitive CRP, *APOE* = Apolipoprotein E, e2/e2 or e2/e3 = apolipoprotein Eε2/ε2 genotype, e3/e3 = apolipoprotein Eε3/ε3 genotype, e3/e4 or e4/e4 = apolipoprotein Eε3/ε4 or ε4/ε4 genotype.

4.3 Telomere length and the risk of dementia

ABSTRACT

There is a wide interest in biomarkers that capture the burden of detrimental factors as these accumulate with the passage of time, i.e., increasing age. Telomere length has received considerable attention as such a marker, because it is easily quantified and it may aid in disentangling the aetiology of dementia or serve as predictive marker. We determined the association of telomere length with risk of Alzheimer disease and all-cause dementia in a population-based setting. Within the Rotterdam Study, we performed quantitative PCR to measure mean leukocyte telomere length in blood. We determined the association of telomere length with risk of Alzheimer's disease until 2016, using Cox regression models. Of 1,961 participants (mean age 71.4±9.3 years, 57.1% women) with a median follow-up of 8.3 years, 237 individuals were diagnosed with Alzheimer's. We found a U-shaped association between telomere length and risk of Alzheimer's: compared to the middle tertile the adjusted hazard ratio was 1.59 (95% confidence interval (CI), 1.13-2.23) for the lowest tertile and 1.47 (1.03-2.10) for the highest tertile. Results were similarly U-shaped but slightly attenuated for all-cause dementia. In conclusion, shorter and longer telomere length are both associated with an increased risk of Alzheimer's disease in the general population.

Keywords: Dementia, Alzheimer disease, telomere, population-based, prospective cohort study

INTRODUCTION

Dementia is a devastating disease affecting millions of people worldwide. While advances over the past decades have unravelled several pathological mechanisms underlying dementia, chronological age remains the most important predictor for dementia onset (Collaborators, 2018). There is, therefore, a wide interest in biomarkers that capture the burden of detrimental factors as these accumulate with the passage of time, i.e., increasing age. Such markers, that are preferably easily quantified, may aid in disentangling the aetiology of dementia or ultimately serve as predictive markers. Against this background, telomere length has received considerable attention (Blackburn *et al.*, 2015).

Telomeres are repetitive base pair sequences at the end of chromosomes and facilitate complete chromosome replication (Martinez and Blasco, 2015). Since the replication machinery is unable to copy the ends of DNA, telomeres will shorten with each cell division. A change in telomere length resulting in shortened telomeres induces a DNA damage response that leads to a growth arrest during which cells attempt to repair the damage and if DNA damage is irreparable, replicative senescence or cell death is triggered (Martinez and Blasco, 2018). Very short telomeres have relatively low cancer risk (due to cell senescence blocking cell division), but could be associated with increased DNA damage (Min *et al.*, 2017). Given the tight link with cell death, short telomere length has been linked to poorer survival (Fasching, 2018; Wang *et al.*, 2018) and other age-related diseases, including dementia as has been shown in a recent meta-analysis (Forero *et al.*, 2016). Conversely, there is evidence that over-elongated telomeres represent pathological cell function with decreased DNA repair and increased cancer risk (Zhang *et al.*, 2017), which has also been shown in human embryonic stem cells (Rivera *et al.*, 2017), while "normally" long telomeres are protective, including against DNA damage. With respect to Alzheimer's disease, there is also evidence suggesting an increased risk of mild cognitive impairment related to both short and long telomeres (Roberts *et al.*, 2014). Additionally, hippocampal cells of Alzheimer's disease brains are shown to have longer telomeres compared to control samples (Thomas *et al.*, 2008). It is, thus, conceivable that not only shorter but also longer telomere length may be detrimental for dementia risk. However, the temporal relation between longer telomere length and dementia has so far not been investigated. Therefore, the aim of this study is to investigate the association between blood cell telomere length and risk of Alzheimer's disease and to explore non-linearity within this association.

METHODS

Design and study population

This study was embedded within the Rotterdam Study, a prospective population-based cohort study among middle-aged and elderly individuals in the Netherlands. Three cohorts were established in 1990, 2000 and 2006, respectively. From each cohort, participants were invited every 4-5 years to undergo follow-up examinations. We refer the reader to the Rotterdam Study methods paper for further information (Ikram *et al.*, 2017). For this study, a total of 2,140 participants were randomly selected from the first (1990-2004, N=7983) and third cohorts (2006-2008, N=3932) for telomere length measurement. From these participants, 1,961 were dementia-free at baseline visit and had complete telomere length data available at baseline (**Figure 1**).

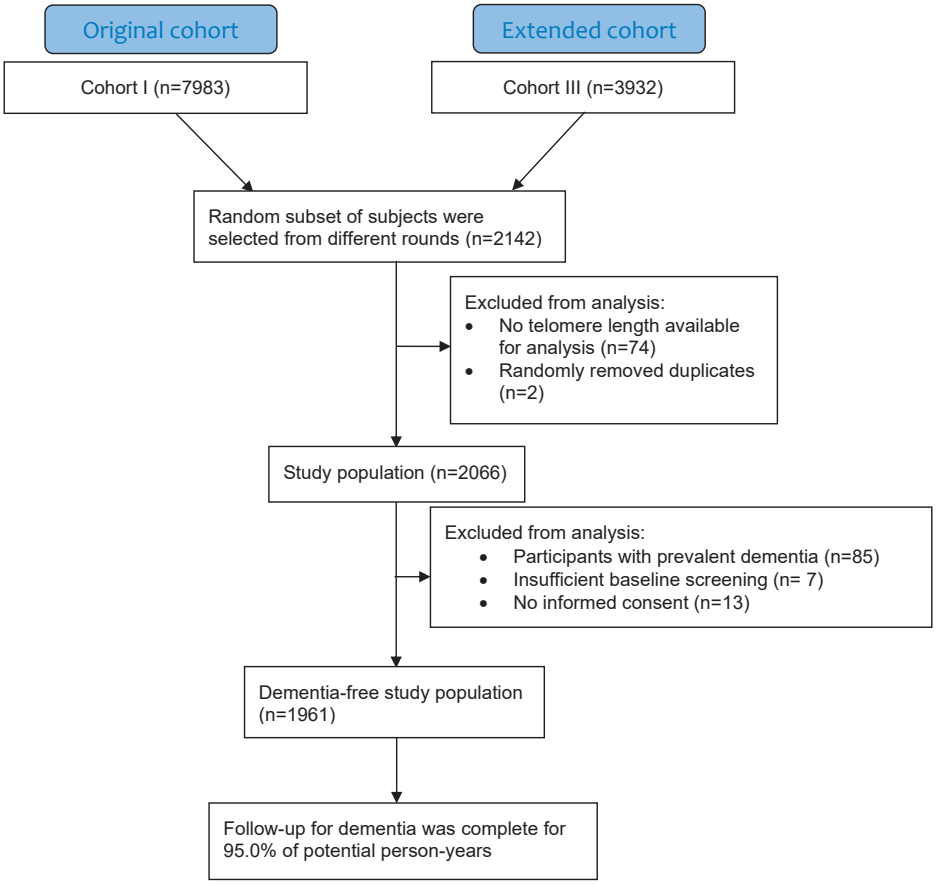

Figure 1. Flow-chart of T/S ratio study population for dementia.

The Rotterdam Study has been approved by the Medical Ethics Committee of the Erasmus MC and by the Ministry of Health, Welfare and Sport of the Netherlands, implementing the Wet Bevolkingsonderzoek: ERGO (Population Studies Act: Rotterdam Study). All participants provided written informed consent to participate in the study and to obtain information from their treating physicians.

Assessment of telomere length

Blood for all samples was collected in EDTA tubes and stored at -20°C until DNA isolation. DNA isolation was done with a method based on salting out, with different lysis buffers for the different cohorts. The OD260/280 and OD260/230 for both isolation methods did not differ significantly between the two cohorts. The average OD260/280 ratio was 1.90 with a standard deviation of 0.10 and the average OD260/230 ratio was 1.61 with a standard deviation of 0.46 in a random sample of 168.Telomere length was measured using a qPCR assay based on the method described by Cawthon (Cawthon, 2002) with minor modifications. The SDS software of the 7900HT qPCR machine is not able to analyse the qPCR data produced at two different temperature points. Therefore we chose the singleplex assay for this study. For each sample the telomere and 36B4 assay were run in separate wells but in the same 384 wells PCR plate. Each reaction contained 5 ng DNA, 1 uM of each of the telomere primers (tel1b-forward: GGTTTGTTTGGGTTTGGGTTTGGGTTTGGGTTTGGGTT, tel2b-reverse: GGCTTGCCTTACCCTTACCCTTACCCTTACCCTTACCCT) or 250 nM of the 34B4 primers (36B4u-forward: CAGCAAGTGGGAAGGTGTAATCC, 36B4d-reverse: CCCATTCTATCATCAACGGGTACAA) and 1x Quantifast SYBR green PCR Mastermix (Qiagen). The reactions for both assays were performed in duplicate for each sample in a 7900HT machine (Applied Biosystems). Ct values and PCR efficiencies were calculated per plate using the MINER algorithm (Codd *et al.*, 2010). Duplicate Ct values that had a Coefficient of Variance (CV) of more than 1% were excluded from further analysis. Using the average Ct value per sample and the average PCR efficiency per plate the samples were quantified using the formula Q=1/(1+PCR eff)^Ct. The relative telomere length was calculated by dividing the Q of the telomere assay by the Q of the 34B4 assay. To validate the assay 96 random samples were run twice and the CV of that experiment was 4.5%.Telomere length was measured in 37 batches.

Ascertainment of incident dementia

Baseline and follow-up ascertainment methods for dementia have previously been described in detail (de Bruijn *et al.*, 2015). In brief, participants were screened for dementia at baseline and subsequent center visits. In addition, the entire cohort was continuously under surveillance for dementia through electronic linkage of the study database with medical records. A consensus panel led by a consultant neurologist established the final diagnosis according to standard criteria for dementia (DSM-III-R) and Alzheimer's disease (NINCDS–ADRDA).

Within this period, participants were censored at date of dementia diagnosis, death, loss to follow-up, or 1st of January 2016, whichever came first.

APOE genotyping

APOE genotype was determined using polymerase chain reaction on coded DNA samples in the baseline cohort (Slooter *et al.*, 1998) and with a bi-allelic Tacqman assay (rs7412 and rs429358) in the extensions of the Rotterdam Study (Ikram *et al.*, 2017). *APOE*-ε4 carrier status was defined carriers versus non-carriers of the ε4 allele.

Other covariates

Potential confounding factors were chosen on the basis of previous literature (de Bruijn *et al.*, 2015; Nilsonne *et al.*, 2015). All covariates were measured at baseline. Educational attainment was categorized as low (primary education or lower vocational education), intermediate (secondary education or intermediate vocational education), and high educational level (higher vocational education or university). Smoking habits were categorized as current, former and never smoking. BMI was calculated as weight in kilograms per height in meters squared. Blood pressure was measured twice at the right brachial artery with the participant in sitting position, out of which the mean was used. Hypertension was defined as a resting blood pressure exceeding 140/90 mmHg or the use of blood pressure lowering medication. Hypercholesterolemia was defined as a total cholesterol of ≥6.2 mmol/L or the use of serum lipid-reducing agents. White blood cell counts were assessed with a Coulter AcT diff2 Hematology Analyzer at the research centre in Ommoord. At 14 February 2005 a new device of the same brand and type was installed. Prevalent stroke was assessed at baseline during interview and we verified these data with medical records. After study entry, we continuously monitored participants after enrollment for incident stroke through linkage of the study database with files from general practitioners. Files of nursing home physician's and files from general practitioners of participants who moved out of the district were also checked. We obtained additional information from hospital records. When potential strokes were found, they were reviewed by research physicians and verified by an experienced neurologist.

Statistical analysis

Missing covariate data (maximum 2.2%) were imputed using 5-fold multiple imputation based on determinant, outcome, and included covariates. In all models, we adjusted for age continuously, sex and center visit since data was randomly selected from 4 center visits from 2 subcohorts. We additionally adjusted for educational attainment, *APOE*-ε4 carrier status, smoking, body mass index (BMI), hypertension, hypercholesterolemia and white blood cell count in a second model, all at time of blood draw. We first explored linearity of telomere length with Alzheimer's disease and dementia risk by entering T/S ratio as restricted cubic spline with 3 knots in Cox regression models. In order to further quantify potential

non-linearity, T/S ratio was subsequently divided into tertiles, defined as T/S ratio of 0.31-0.87, 0.87-1.02 and 1.02-1.79, respectively. To provide a parsimonious description of the telomere-dementia relationship and facilitate communication of the results, we assessed the association between telomere length in tertiles, comparing lowest and highest tertiles that represent the shortest and longest telomere lengths, respectively, to the middle tertile, with risk of Alzheimer's disease and dementia. We repeated the analysis for Alzheimer's disease, by adjusting not only for age at blood draw, but also for age at dementia diagnosis or date of last follow-up as an attempt to assess survival bias. We further verified that age was robustly controlled for by comparing results across models using five different ways of age-adjustment. These included no adjustment for age, entering age and age^2 in the model, entering age as cubic splines, repeating the analyses with age rather than follow-up time as the time scale and finally regressing the effect of age out of T/S ratio before creating T/S ratio tertiles. We performed an additional adjustment for plate number to rule out batch effects. The proportional hazards assumption was assessed using Schoenfeld residuals. We next repeated the analyses for AD, but excluding all participants with prior clinical stroke at baseline and censoring for incident clinical stroke during follow-up using Cox models.

We also assessed interaction by age, sex, education or *APOE*-ε4 carrier status by stratification and testing for multiplicative interaction by including the product of the interacting factor and T/S ratio continuously as a restricted cubic spline (4 knots) to take into account the non-linear relationship between T/S ratio and Alzheimer's disease. We stratified for age by 3 groups, namely 45-65 years, 65-85 years and 85-105 years.

All analyses were performed using RStudio version 0.99.903 (R version 3.4.3(Team, 2017), RStudio, Inc., Boston, MA). We used the following R packages for our analyses: mice, survival, rms and scales.

RESULTS

Baseline characteristics of the study population are presented in **Table 1**. The mean age of the participants was 71.4±9.3 years with 57.1% women. During a median follow-up of 7.3 (interquartile range 4.9-11.7) years (26,025 person-years), 305 individuals were diagnosed with dementia of whom 237 were Alzheimer's disease.

Table 1. Baseline characteristics of the dementia-free study population.

Characteristics	**Dementia-free cohort** (N = 1961)
Women	1120 (57.1%)
Age, years	71.4 ±9.3
Cohort	
First	1543 (78.7%)
Third	418 (21.3%)
Education	
Primary education	366 (18.9%)
Lower/intermediate general education	806 (41.5%)
Intermediate vocational education	535 (27.6%)
Higher vocational education	233 (12.0%)
Apolipoprotein E4ε carriership	
Non-carrier (ε2/ε2 , ε2/ε3 or ε3/ε3)	1411 (72.0%)
Carrier (ε4/ε4, ε3/ε4 or ε2/ε4)	550 (28.0%)
Smoking	
Current	405 (21.1%)
Former	921 (48.0%)
Never	592 (30.9%)
BMI, kg/m^2	27.2 ±4.1
Hypertension	700 (36.5%)
Hypercholesterolemia	949 (48.8%)
White blood cell count	6.9 ±2.3
Telomere length, T/S ratio	0.95 ±0.18

Abbreviations: N = number of participants included in study. Data presented as mean (standard deviation) for continuous variables and number (percentages) for categorical variables. T/S ratio is relative telomere to single copy gene ratio. Data represent original data without imputed values. Number of missing values are 43 (2.2%) for smoking, 21 (1.1%) for education, 27 (1.4%) for hypertension, 17 (0.9%) for hypercholesterolemia, 29 (1.5%) for body mass index and 157 (8.0%) for white blood cell count. Data for apolipoprotein E4ε carriership was complete.

There was evidence of non-linearity in the association between telomere length and Alzheimer's disease; **Figure S1**. Compared to the middle tertile of telomere length, we found an adjusted hazard ratio (aHR) of 1.59; 95% confidence interval (CI), 1.13-2.23 for lowest tertile of telomere length and 1.47; 95%CI, 1.03-2.10 for highest tertile of telomere length for Alzheimer's disease, with similar but lower estimates for dementia (**Table 2)**. When additionally adjusting for age at dementia diagnosis or date of last follow-up, we found an aHR of 1.52; 95%CI, 1.07-2.18 for lowest tertile of telomere length and 1.75; 95%CI, 1.21-2.54 for highest tertile of telomere length for Alzheimer's disease, compared to the middle tertile. Other approaches of age-adjustment yielded similar results (**Table 3**). There were no batch effects. The proportional hazards assumption was met for all models. Results were slightly attenuated but remained U-shaped after excluding participants with prior clinical stroke at baseline and censoring for incident clinical stroke: compared to middle tertile aHR: 1.44, 1.02-2.05 for lowest tertile of telomere length and aHR 1.30, 95% CI 0.90-1.89 for highest tertile of telomere length.

Table 2. Telomere length and the risk of Alzheimer's disease and all-cause dementia.

	n/N	Alzheimer's disease		n/N	All-Cause dementia	
		Model I HR, 95% CI	Model II HR, 95% CI		Model I HR, 95% CI	Model II HR, 95% CI
Telomere length						
Tertile 1 (T/S ratio 0.31-0.87)	106/654	1.54, 1.10 – 2.15	1.59, 1.13 – 2.23	129/654	1.25, 0.94 – 1.66	1.27, 0.96 – 1.70
Tertile 2 (T/S ratio 0.87-1.02)	54/654	1 (reference)	1 (reference)	80/654	1 (reference)	1 (reference)
Tertile 3 (T/S ratio 1.02-1.79)	77/653	1.41, 0.99 – 2.00	1.47, 1.03 – 2.10	96/653	1.19, 0.88 – 1.60	1.25, 0.92 – 1.69

Abbreviations: n = number of cases; N = number of persons at risk; HR = hazard ratio; CI = confidence interval; T/S ratio = relative telomere to single copy gene ratio. Cox regression model I: Adjusted for age, sex and visit. Cox regression model II: Adjusted for age, sex, visit, education, *APOE*-ε4 carrier status, smoking, BMI, hypertension, hypercholesterolemia and white blood cell count.

In the stratified analyses, the association between shorter and longer telomeres and Alzheimer's disease was stronger in *APOE*-ε4 carriers compared to non-carriers (**Figure 2**) with the multiplicative interaction term yielding p=0.087. Stratification for age, sex and education did not provide different risk estimates (P-values for interaction: p = 0.554, p = 0.600 and p = 0.123, respectively).

Table 3. Association between telomere length and Alzheimer's disease using different modes of age adjustment.

Adjustment	**Alzheimer's disease**					
		No age adjustment	**Age + age^2**	**Cubic splines for age†**	**Age as time-scale**	**Regressing out the effect of age of T/S ratio***
	n/N	HR, 95% CI	HR, 95% CI	HR, 95% CI	HR, 95% CI	HR, 95% CI
Telomere length						
Tertile 1 (shortest)	106/654	2.08, 1.49-2.91	1.64, 1.17-2.30	1.64, 1.17-2.30	1.63, 1.16-2.29	1.33, 0.96-1.86
Tertile 2 (middle)	54/654	1 (reference)	1 (reference)	1 (reference)	1 (reference)	1 (reference)
Tertile 3 (longest)	77/653	1.25, 0.88-1.77	1.45, 1.01-2.06	1.45, 1.02-2.06	1.44, 1.01-2.06	1.22, 0.87-1.69

Abbreviations: n = number of cases; N = number of persons at risk; HR = hazard ratio; CI = confidence interval; T/S ratio = relative telomere to single copy gene ratio. †Cubic splines with 3 knots, similar results for higher degree polynomials.

Models adjusted for age, sex, visit, education, *APOE*-ε4 carrier status, smoking, BMI, hypertension, hypercholesterolemia and white blood cell count.*Regressing the effect of age out of T/S ratio before creating T/S ratio tertiles; no additional adjustment for age in the model; similar results for additionally regressing out the effect of sex. *Regressing the effect of age out of T/S ratio before creating T/S ratio tertiles; similar results for additionally regressing out the effect of sex.

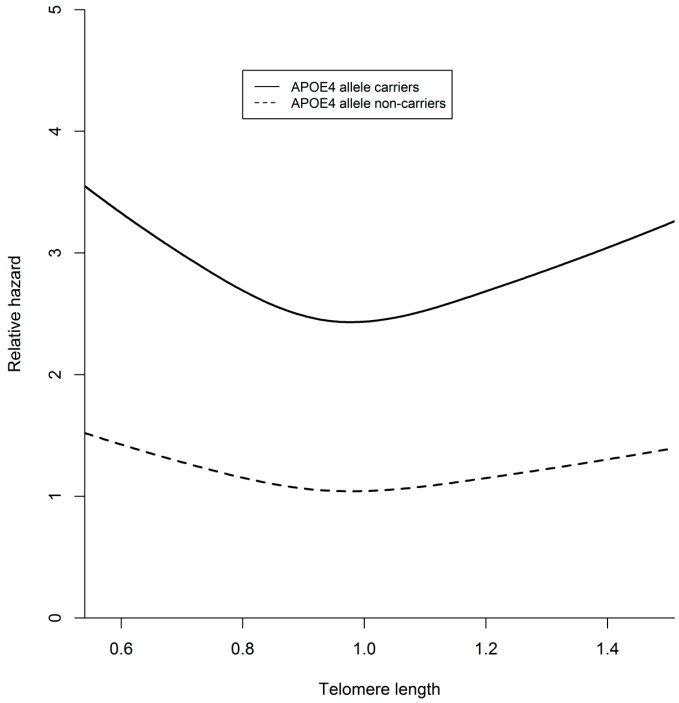

Figure 2. The association between telomere length and the risk of Alzheimer's disease stratified for APOE4 allele carriers and non-carriers.
A visual representation of telomere length and the risk of Alzheimer's disease (AD) stratified by APOEε4 carriership with restricted cubic splines in Cox model II, adjusted for age, sex, study visit, education, APOEε4 carrier status, smoking, BMI, hypertension, hypercholesterolemia and white blood cell count. Number of AD cases in APOEε4 carrier group: 102 with 550 persons at risk; number of cases in the APOEε4 non-carrier group: 135 with 1411 persons at risk.

DISCUSSION

In this study, shorter as well as longer telomere length were significantly associated with increased risk of dementia, specifically Alzheimer's disease.

Thus far, focus in the dementia field has been on shorter telomere length with studies finding observationally as well as genetically associations between shorter telomere length and increased risk of AD (Honig *et al.*, 2012; Zhan *et al.*, 2015; Guo and Yu, 2019; Scheller Madrid *et al.*, 2019). Moreover, a recent meta-analysis which included 13 primary studies demonstrated significantly shorter telomere length in 860 AD patients compared to 2,022 controls (Forero *et al.*, 2016). Interestingly, we found that in addition to shorter telomere length also longer telomere length was associated with an increased dementia risk. This

finding is in line with previous studies (Wikgren *et al.*, 2012a; Wikgren *et al.*, 2012b), particularly the study by Roberts and others (Roberts *et al.*, 2014) showing that short and long telomeres increase risk of amnestic mild cognitive impairment. Yet, we emphasize that further replication is necessary to confirm the robustness of our findings with dementia.

An explanation for our observations with longer telomere length might be survival bias, where presumably participants with the longest telomere length live long enough to develop dementia. This is however less likely since associations remained after additional adjustment for age at dementia diagnosis or end of follow-up. Alternatively, it is plausible that a disturbed equilibrium of telomere length towards either direction is harmful. Indeed, over-elongated telomeres become fragile and accumulate DNA damage, as has been shown by an experimental study in human embryonic stem cells, and very tight control of telomere length homeostasis is regulated by not only telomerase-dependent elongation, but also by telomere trimming events (Rivera *et al.*, 2017). Another study has shown that telomerase RNA component (TERC) knockout mice with AD, which present with telomere shortening, slows down the progression of Aβ pathology, showing a more protective effect of short telomere length (Rolyan *et al.*, 2011), thereby supporting a detrimental effect of longer telomere length. The potential biological link between over-elongated telomeres and higher AD risk remains to be further elucidated. In contrast with longer telomere length, more is known about the potential biological link between shorter telomeres and increased AD risk. Neurons lacking telomerase reverse transcriptase (TERT) in knockout mice and thus have shorter telomeres, display an increased production of oxidative species and an increase in cellular oxidative damage in response to tau, demonstrating a harmful effect of shorter telomeres (Spilsbury *et al.*, 2015). Therefore, telomerase may play different roles in the tau and amyloid pathology via multiple mechanisms (Liu *et al.*, 2018). In addition, microglial cellular senescence may also be an important mechanism in the development of AD, which is suggested to be exacerbated by the presence of amyloid (Flanary, 2005; Flanary *et al.*, 2007). Further studies are warranted to differentiate between a true biological association and survival bias. If a true biological association exists between longer telomere length and risk of dementia, telomerase therapies, which have been successful in mice (Jaskelioff *et al.*, 2011; Bernardes de Jesus *et al.*, 2012), would need further fine-tuning to prevent over-elongation of telomere length.

Interestingly, we found stronger effects for Alzheimer's disease compared to all-cause dementia. This might be reflective of the fact that Alzheimer's disease is a more homogeneous entity than all-cause dementia, which includes a plethora of etiologies.

Certain limitations must be taken into account. First, we measured telomere length in leukocytes in blood. This may not be representative of telomere length in the brain, in particular glial cells. However, a previous study has shown a direct correlation between telomeres measured from peripheral blood and cerebellum (Lukens *et al.*, 2009), suggesting blood cell telomere length to be a valid measurement. Another limitation of measuring telo-

mere length in leukocytes is that lymphocytes are the only peripheral blood cells expressing telomerase activity and therefore are able to maintain telomere length despite proliferation, while granulocytes lack the ability for further cell division (Akbar and Vukmanovic-Stejic, 2007). Therefore, it is possible that the older individuals without dementia have a greater proportion of lymphocytes (fewer granulocytes) (van der Willik *et al.*, 2019), and thus longer telomere length. Unfortunately, we were not able to adjust for the percentage of lymphocytes to overcome this issue, since this data was not available for all center visits. Another issue is that leukocytes divide peripherally in response to various stresses (e.g., infection) and the telomere length may therefore change unexpectedly. Also, research centers may vary in reliability in measuring leukocyte telomere length. Second, we measure mean telomere length, while cell senescence is suggested to be related to and modulated by the shortest telomere length per cell (Hemann *et al.*, 2001). Third, the external validity of the results may be limited due to the single centre nature of this study. Finally, despite using different approaches yielding similar results, the effect of age may not have been taken into account entirely. Nevertheless, given the link of older age with shorter telomere length and older age with Alzheimer's disease, controlling for age would if anything strengthen the association between longer telomere length and risk of Alzheimer's disease.

In conclusion, shorter and longer telomere length are associated with an increased risk of Alzheimer's disease in a sample of elders in The Netherlands. Further studies are warranted to confirm our findings, in particular studies with imaging or fluid biomarkers available for dementia diagnosis, and to unravel any underlying biological pathway from possible methodological bias.

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SUPPORTING INFORMATION

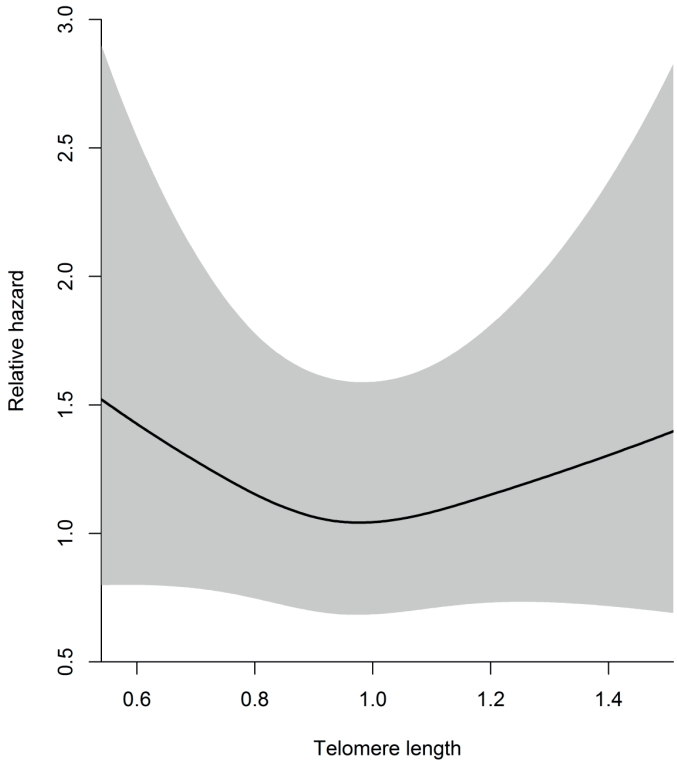

Figure S1. The association between telomere length and the risk of Alzheimer's disease.
A visual representation of telomere length and the risk of Alzheimer's disease (AD) with restricted cubic splines in Cox model II, adjusted for age, sex, study visit, education, APOEε4 carrier status, smoking, BMI, hypertension, hypercholesterolemia and white blood cell count. The U-shaped association between telomere length and Alzheimer's disease risk is clearly visible, showing elevated risk for both shorter and longer telomere length. The black line indicates the hazard ratio and gray area indicates the 95% confidence interval.

Chapter 5
Brain hemodynamics

5.1 Global brain perfusion and the risk of transient ischemic attack and ischemic stroke

ABSTRACT

Background – The role of subtle disturbances of brain perfusion in the risk of TIA or ischemic stroke remains unknown. We examined the association between global brain perfusion and risk of TIA and ischemic stroke in the general population.

Methods and Results - Between 2005-2015, 5289 stroke-free participants (mean age 64.3 years, 55.6% women) from the Rotterdam Study underwent phase-contrast brain magnetic resonance imaging at baseline to assess global brain perfusion. These participants were followed for incident TIA or ischemic stroke until January 1st, 2016. We investigated associations between global brain perfusion (mL blood flow/100mL brain/minute) and risk of TIA and ischemic stroke using Cox regression models with adjustment for age, sex, and cardiovascular risk factors. Additionally, we investigated whether associations were modified by retinal vessel calibers, small and large vessel disease, blood pressure and heart rate. During a median follow-up of 7.2 years (36,103 person-years), 137 participants suffered a TIA and another 108 an ischemic stroke. We found that lower global brain perfusion was associated with a higher risk of TIA but not with the risk of ischemic stroke (adjusted hazard ratio (aHR), 95% confidence interval (CI), per standard deviation decrease of global brain perfusion: 1.29, 1.07–1.55 for TIA and aHR of 1.06, 0.87–1.30 for ischemic stroke). Across strata of wider arteriolar retinal calibers, lower brain perfusion was more prominently associated with TIA, but not with ischemic stroke.

Conclusions - In a community-dwelling population, impaired global brain perfusion increased the risk of TIA but not of ischemic stroke.

INTRODUCTION

Stroke is caused by insufficient perfusion to specific regions of the brain, leading to neuronal dysfunction and ultimately cell death[1]. If the disruption is of short duration, it may instead lead to a transient ischemic attack (TIA) [2]. Maintenance of sufficient perfusion of the brain is achieved primarily by arterioles, which either dilate or contract[3]. In the presence of cardiovascular risk factors and arteriolosclerosis this regulating ability of arterioles is affected and can lead to disruptions in brain perfusion. In this light, cerebral hypoperfusion has been suggested as a potential link between vascular damage and TIA and stroke and is a potential target for preventive interventions [4, 5]. However, the temporal relationship between cerebral hypoperfusion and the risk of TIA or stroke remains undefined. Global brain perfusion is a hemodynamic factor, which reflects more subtle and chronic changes in brain perfusion[6]. Until recently, studies assessing global brain perfusion have only been performed in people with previous stroke or in patients with asymptomatic or symptomatic high-grade carotid stenosis, in which lower global brain perfusion increased the risk of stroke[7]. Yet, it is unknown whether in the absence of high-grade carotid stenosis global brain perfusion is related to the occurrence of TIA and stroke. Moreover, it is unclear whether any impact of poor global brain perfusion on stroke risk is augmented by additional pathology along the brain vasculature, either as large vessel or small vessel disease. Identifying individuals who are at increased hemodynamic risk of stroke can be of major importance for preventive purposes. Against this background, we investigated the association of global brain perfusion at baseline with the risk of TIA and ischemic stroke in a large sample of community-dwelling elderly with an average follow-up of 7 years. We hypothesized that lower global brain perfusion is a risk factor for incident TIA and ischemic stroke and that this association is modified by markers of small and large vessel disease.

METHODS

Study population

This study is embedded within the Rotterdam Study, a large population-based cohort study in the Netherlands that started in 1990. The study is aimed at investigating determinants of various chronic diseases among elderly people[8]. The original study population consisted of all residents of Ommoord, a district in Rotterdam, aged 55 years and older. In 2000, the cohort was expanded with another 3,011 participants with the same inclusion criteria and in 2006, a further expansion of the cohort was initiated in which 3,932 participants were included, aged 45-54 years. Follow-up examinations at the research center are performed every 3 to 5 years. From 2005 onwards, brain MRI was implemented in the core study protocol and all participants were invited to undergo brain MRI[9].

The Rotterdam Study has been approved by the Medical Ethics Committee of the Erasmus MC (registration number MEC 02.1015) and by the Dutch Ministry of Health, Welfare and Sport (Population Screening Act WBO, license number 1071272-159521-PG). The Rotterdam Study has been entered into the Netherlands National Trial Register (NTR; www.trialregister.nl) and into the WHO International Clinical Trials Registry Platform (ICTRP; www.who.int/ictrp/network/primary/en/) under shared catalogue number NTR6831. All participants provided written informed consent to participate in the study and to have their information obtained from treating physicians. Requests to access the dataset from qualified researchers trained in human subject confidentiality protocols may be sent to Dept. of Epidemiology, Erasmus MC University Medical Center at f.vanrooij@erasmusmc.nl.

Population for analysis

Of 5,644 persons undergoing a first MRI scan, no reliable measure of cerebral blood flow could be obtained in 5 (0.09%) persons. After excluding participants with prevalent TIA or stroke (n = 320) and 30 participants with missing follow-up (n = 30) data, 5,289 participants were included for analysis. Follow-up for TIA and stroke was complete for 93.7% of potential person-years (Figure 1).

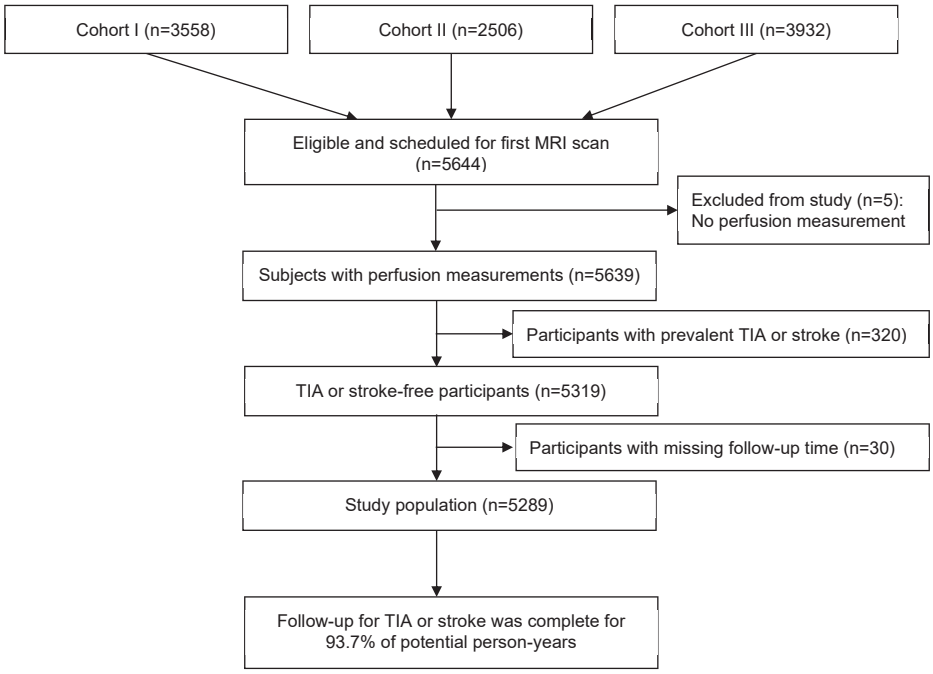

Figure 1. Flow-chart of study population.

Assessment of global brain perfusion

All participants were scanned at baseline on a 1.5-Tesla MRI scanner (General Electric Healthcare, Milwaukee, WI, USA) with a dedicated eight-channel head coil. The full MRI protocol has been described in detail before[9]. Specifically, for the flow measurements, we performed a 2D phase-contrast sequence[10, 11]. A 2D thick slab projection phase contrast angiographic localizer (60 mm thick, velocity encoding (VENC) = 60 cm/sec) is positioned sagittally in order to determine the location of the carotid and basilar arteries. Next, a thin slice perpendicular to all three vessels at the level of the precavernous internal carotid artery is positioned (VENC = 120 cm/s, slice thickness 5 mm, NEX = 8). From the resulting images, we calculated cerebral blood flow using interactive data language-based custom software (Cinetool version 4, General Electric Healthcare, Milwaukee, WI, USA). Two independent, experienced technicians drew circular to elliptical regions of interest (ROIs) manually around both carotids and the basilar artery at the level of the clinoid segment on the phase-contrast images, encompassing the entire lumen of the vessel (inter-rater correlations > 0.94 for all vessels)[12]. For improved visualization of vessel boundaries, the contrast between the arteries of interest and the background were inverted. In each ROI, the value of mean signal intensity reflected the flow velocity in the vessel (cm/sec). By multiplying the average velocity with the cross-sectional area of the vessel, flow (in mL/sec) was calculated. To obtain global cerebral blood flow (tCBF) in mL/min, flow rates for the carotid arteries and the basilar artery were summed and multiplied by 60 secs/min. We calculated global brain perfusion (in mL/min blood per 100 mL brain) by dividing tCBF (mL/min) by each individual's brain volume (mL) and multiplying the obtained result by 100 mL brain volume. Brain volume was calculated by adding up gray and white matter volumes, converted to millilitres.

Assessment of retinal vessel calibers

Retinal microvascular calibers are considered markers of small vascular pathology, but also markers of vascular autoregulation[13, 14], which we therefore stratified for. We assessed retinal microvascular calibers (arteriolar and venular calibers (in µm)) using fundus photographs and the Retinal Vessel Measurement System (Retinal Analysis, Optimate, WI; Department of Ophthalmology and Visual Science, University of Wisconsin-Madison)[15] as previously described[16, 17]. Retinal microvascular calibers were assessed in a subset of 3219 individuals.

Assessment of small vessel and large vessel disease

For the lacunar infarcts and WMHs measurements on MRI scans, gray matter volume (GM), white matter volume (WM) and cerebrospinal fluid were quantified using an automated tissue segmentation method, based on a k-nearest-neighbour brain tissue classifier algorithm[18]. This was extended with a custom-developed WMH (white matter hyperintensity) segmentation[19]. Segmentation results were visually inspected and if needed corrected manually. All scans were furthermore rated by trained research physicians, blinded to clinical data, for the

presence of lacunar infarcts (defined as focal lesions ≥3 and <15 mm in size with the same signal characteristics as cerebrospinal fluid on all sequences, and, if located supratentorially, with a hyperintense rim on the fluid attenuated inversion recovery sequence).

Markers of large vessel disease (intima-media thickness and carotid plaques) were measured as follows: Doppler ultrasound was used to measure carotid stenosis (≥50% stenosis). To measure carotid intima-media thickness and carotid plaques, ultrasonography of the common carotid artery, carotid bifurcation, and left and right internal carotid artery was performed with a 7.5-MHz linear-array transducer (ATL Ultra-Mark IV)[20].

Stroke and TIA assessment

The definition of stroke was based on the WHO criteria[21], describing a syndrome of rapidly developing symptoms of focal or global cerebral dysfunction lasting 24 hours or longer or leading to death, with apparent vascular cause. Prevalent stroke was assessed at baseline during interview and we verified these data with medical records. After study entry, we continuously monitored participants after enrollment for incident stroke through linkage of the study database with files from general practitioners. Files of nursing home physician's and files from general practitioners of participants who moved out of the district were also checked. We obtained additional information from hospital records. When potential strokes were found, they were reviewed by research physicians and verified by an experienced neurologist. We then categorized strokes into ischemic or haemorrhagic on the basis of neuro-imaging reports. If neuroimaging was missing and we could not differentiate the type of stroke according to the symptoms, the stroke was classified as unspecified. Subarachnoid haemorrhages due to ruptured aneurysms were not considered stroke events. For this study, we were mainly interested in ischemic stroke as outcome. Follow-up for incident stroke was complete until January 1st 2016.

A similar work-up was performed to determine prevalent and incident TIA[22]. *Transient neurological attacks (TNA)* were defined as attacks of sudden neurological symptoms that completely resolved within 24 hours, with no clear evidence for the diagnosis of migraine, epilepsy, Meniere disease, hyperventilation, cardiac syncope, hypoglycemia, or orthostatic hypotension[23]. If only focal brain symptoms (hemiparesis, hemihypesthesia, dysphasia, dysarthria, amaurosis fugax, hemianopia, hemiataxia, diplopia, or vertigo) were reported, the event was classified as a *focal TNA*. If only nonfocal brain symptoms (decreased consciousness, unconsciousness, confusion, amnesia, unsteadiness, nonrotatory dizziness, positive visual phenomena, cardiac or vegetative signs, paresthesias, bilateral weakness, and unwell feelings) were reported, the event was classified as *nonfocal TNA*. If both focal and nonfocal symptoms were reported for the same attack, a *mixed TNA* was diagnosed. For this study, we only used the focal and mixed TNAs as outcome for TIA.

Other measurements in the Rotterdam Study

Information on cardiovascular risk factors was obtained by interview, physical examination and blood sampling[8]. We assessed history of smoking (current, former, never), and use of antihypertensive, lipid-lowering and anti-thrombotic medication at baseline by interview. Body mass index was calculated as weight (kg)/length (m^2). Diastolic and systolic blood pressures were measured twice on the right arm with a random-zero sphygmomanometer; for analyses the mean of these readings was used. Heart rate was measured during sitting blood pressure measurement. Total cholesterol and high-density lipoprotein (HDL) cholesterol were measured by an automated enzymatic procedure. Diabetes mellitus type 2 was defined as the use of blood glucose-lowering medication at baseline or having a fasting serum glucose level of ≥126 mg/dL or ≥7.0 mmol/l[24].

Statistical analysis

First, we investigated the association of global brain perfusion (per standard deviation decrease, and in tertiles) with the risk of TIA and ischemic stroke separately and combined (whichever came first) using Cox proportional hazards models. In model 1, we adjusted for age, sex and cohort. In model 2 we additionally adjusted for smoking status, systolic and diastolic blood pressure, blood pressure- and lipid lowering-medication, antithrombotic medication use, diabetes mellitus, BMI, carotid stenosis, total cholesterol, HDL and MRI-defined lacunar infarcts. We censored for incident TIAs during follow-up in the analyses on ischemic stroke and for incident stroke in the analyses on TIA. We repeated the same analyses while not censoring for incident TIAs during follow-up in the analyses on stroke to compare what the effect is of brain perfusion when having a TIA before a stroke. For all Cox models, we tested the proportional hazard assumption using Schoenfeld residuals.

Second, we investigated potential effect modification by retinal vessel calibers, small and large vessel disease and finally blood pressure and heart rate on the association of brain perfusion with TIA and ischemic stroke. As markers of small vessel disease, we considered lacunar infarcts and WMH. While lacunar infarcts were dichotomized into yes versus no, we stratified WMH at their median (3.0 mL) and retinal calibers into tertiles. Regarding the association of blood pressure, lacunar infarcts and WMH with global brain perfusion we would like to point to the work of our colleagues in this same sample of community-dwelling people[25, 26]. As markers of large vessel disease, we considered carotid intima media thickness and carotid plaques. While we stratified carotid intima media thickness using a cut-off of 1 mm, carotid plaques were dichotomized into presence and absence. As cardiovascular determinants we chose systolic and diastolic blood pressure and heart rate since these modify the diameter of blood vessels. Due to potential overfitting of the models, we only adjusted these analyses for age, sex and study cohort. In addition to these stratified analyses, we also tested for interaction on the multiplicative scale by adding interaction terms to the further adjusted regression models (model 2).

Third, since we defined TIA and ischemic stroke as clinical diagnoses, we assessed the effect of brain infarction on MRI by performing a sensitivity analysis in which we excluded participants with the presence of cortical infarcts on brain MRI. Finally, we excluded people with a carotid stenosis at baseline to preclude the effect of carotid stenosis on the association between global brain perfusion and TIA or ischemic stroke.

Missing data on covariables (maximum 10.3%) were imputed using 5-fold multiple imputation based on determinant, outcome and covariables with 20 iterations for each imputation. Distribution of covariables was similar in the imputed versus non-imputed dataset.

Analyses were done using RStudio version 1.0.153 (2009-2017 RStudio, Inc).

RESULTS

Of the 5289 stroke-free participants at baseline, mean age was 64.3 years, (standard deviation 10.6) with 55.6% women and a mean global brain perfusion of 56.2 ml/min per 100 mL brain (standard deviation 9.7; Table 1). During a median follow-up of 7.2 years (36,103 person-years), 137 suffered a TIA (mean global brain perfusion 52.7) and 137 a stroke (mean global brain perfusion 53.3), whichever came first (incidence rate for both events: 3.8 per 1000 person-years). Within the stroke group, 108 participants developed ischemic stroke (mean global brain perfusion 53.4), 20 participants developed haemorrhagic stroke and 9 participants developed a stroke which was not further specified. Among participants who developed TIA, 93 participants had only focal symptoms and 44 participants had a mix of focal and non-focal symptoms. The majority developed a single TIA, while 22 participants had multiple attacks.

We found that global brain perfusion at baseline associated with a higher risk of TIA (adjusted hazard ratio (aHR), 95% confidence interval (CI), per standard deviation decrease: 1.30; 1.07–1.57) and not of ischemic stroke (aHR, 1.06; 95% CI, 0.87–1.30); Table 2. Effect estimates were higher for the risk of ischemic stroke when not censoring for previous TIA (aHR 1.09; 95% CI, 0.90 – 1.33). Results for the association for lower global brain perfusion with risk of TIA or any stroke combined are shown in Table 3. The proportional hazard assumption was met for all models.

After stratifying the analyses on retinal vessel diameter, lower global brain perfusion was associated more prominently with TIA across strata of wider arteriolar and wider venular retinal vessel calibers compared to strata of smaller retinal vessel calibers (aHR, 2.04, 95% CI, 1.18–3.50 for arterioles and aHR, 2.30, 95% CI, 1.26–4.17 for venules in the widest strata). For ischemic stroke, there were no differences between strata of retinal vessels (aHR, 1.15, 95% CI, 0.64 – 2.06 for arterioles and aHR, 1.07, 95% CI, 1.72–0.79 for venules in the widest strata); Figure 2. However, within strata of higher diastolic blood pressure, participants had an 51% increased risk of developing an ischemic stroke per SD decrease of global brain perfusion, with the interaction term being borderline significant (p=0.056),

whereas higher diastolic blood pressure did not modify the risk of TIA (p=0.444); Figure 2. For other markers of small and large vessel disease, no significant differences were found across strata for the risk of TIA or ischemic stroke (data not shown).

Table 1. Baseline characteristics of the study population

	Total cohort (N = 5289)
Women	2941 (55.6)
Age, years	64.3 (10.6)
Smoking	
Current	1003 (21.1)
Former	2293 (48.3)
Never	1447 (30.5)
Systolic blood pressure, mmHg	139.4 (21.3)
Diastolic blood pressure, mmHg	82.6 (11.0)
Use of blood-pressure lowering medication	1787 (34.1)
Serum total cholesterol, mmol/l	5.6 (1.1)
Serum high-density lipoprotein cholesterol, mmol/l	1.5 (0.4)
Use of lipid-lowering medication	1227 (23.4)
Diabetes mellitus type 2	506 (9.8)
Body mass index, kg/m^2	27.5 (4.2)
Carotid stenosis	141 (2.7)
Use of antithrombotic medication	878 (16.7)
Presence of lacunar infarcts	325 (6.2)
Global brain perfusion, ml/min per 100 mL brain	56.2 (9.7)

Abbreviations: N = number of participants included in study.
Data presented as mean (standard deviation) for continuous variables and number (percentages) for categorical variables. Data represent original data without imputed values.
Number of missing values are 546 (10.3%) for smoking, 38 (0.7%) for systolic blood pressure, 38 (0.7%) for diastolic blood pressure, 42 (0.8%) for use of blood-pressure lowering medication, 89 (1.7%) for serum total cholesterol, 89 (1.7%) for serum high-density lipoprotein cholesterol, 42 (0.8%) for use of lipid-lowering medication, 114 (2.2%) for diabetes mellitus type 2, 32 (0.6%) for body mass index, 89 (1.7%) for carotid stenosis, 42 (0.8%) for use of antithrombotic medication and 12 (0.2%) for the presence of lacunar infarcts.

We found no evidence for a change in effect of brain perfusion on TIA or ischemic stroke after exclusion of all persons with cortical infarcts (n=66) on the MRI scan (aHR, 1.31, 95% CI, 1.08 – 1.58 for TIA and aHR, 1.07, 95% CI, 0.87–1.31 for ischemic stroke). Finally, after excluding people with carotid stenosis (n=190), also no difference was found in effect of brain perfusion on TIA or ischemic stroke (aHR, 1.24, 95% CI, 1.02 – 1.51 for TIA and aHR, 1.06, 95% CI, 0.86 – 1.31 for ischemic stroke).

Table 2. Global brain perfusion and the risk of TIA and ischemic stroke.

	TIA			Ischemic stroke		
	n/N	**Model I**	**Model II**	**n/N**	**Model I**	**Model II**
		HR, 95% CI	**HR, 95% CI**		**HR, 95% CI**	**HR, 95% CI**
Global brain perfusion (per SD decrease)	137/5289	1.34, 1.10 – 1.62	1.30, 1.07 – 1.57	108/5289	1.09, 0.89 – 1.34	1.06, 0.87 – 1.30
Tertile 1 (19 - 52)	59/1716	1.83, 1.14 – 2.92	1.78, 1.11 – 2.85	49/1716	1.33, 0.81 – 2.18	1.29, 0.78 – 2.12
Tertile 2 (52 - 59)	51/1762	1.84, 1.15 – 2.95	1.74, 1.08 – 2.80	33/1762	1.28, 0.77 – 2.13	1.31, 0.79 – 2.18
Tertile 3 (59 - 154)	27/1811	1 (reference)	1 (reference)	26/1811	1 (reference)	1 (reference)

Global brain perfusion tertiles presented as lowest, middle, highest (mL/min per 100 mL).

Abbreviations: n = number of cases; N = number of persons at risk; HR = hazard ratio; CI = confidence interval.

Cox regression model I: Adjusted for sex, age and study cohort.

Cox regression model II: As model I, additionally adjusted for systolic blood pressure, diastolic blood pressure, blood pressure lowering medication, serum total cholesterol, serum high-density lipoprotein cholesterol, lipid-lowering medication, smoking, diabetes mellitus type 2, body mass index, carotid stenosis, antithrombotic medication use, and silent brain infarcts (lacunar infarcts).

Table 3. Global brain perfusion and the risk of TIA or ischemic stroke combined.

	TIA or ischemic stroke		
	n/N	Model I	Model II
		HR, 95% CI	HR, 95% CI
Global brain perfusion (per SD decrease)	246/5289	1.22, 1.06 – 1.40	1.18, 1.03 – 1.36
Tertile 1 (19 - 52)	108/1716	1.55, 1.10 – 2.17	1.49, 1.06 – 2.09
Tertile 2 (52 - 59)	84/1762	1.46, 1.03 – 2.05	1.45, 1.03 – 2.05
Tertile 3 (59 - 154)	54/1811	1 (reference)	1 (reference)

Total brain perfusion tertiles presented as lowest, middle, highest (mL/min per 100 mL).
Abbreviations: n = number of cases; N = number of persons at risk; HR = hazard ratio; CI = confidence interval.
Cox regression model I: Adjusted for sex, age and study cohort.
Cox regression model II: As model I, additionally adjusted for systolic blood pressure, diastolic blood pressure, blood pressure lowering medication, serum total cholesterol, serum high-density lipoprotein cholesterol, lipid-lowering medication, smoking, diabetes mellitus type 2, body mass index, carotid stenosis, anti-thrombotic medication use, and silent brain infarcts (lacunar infarcts).

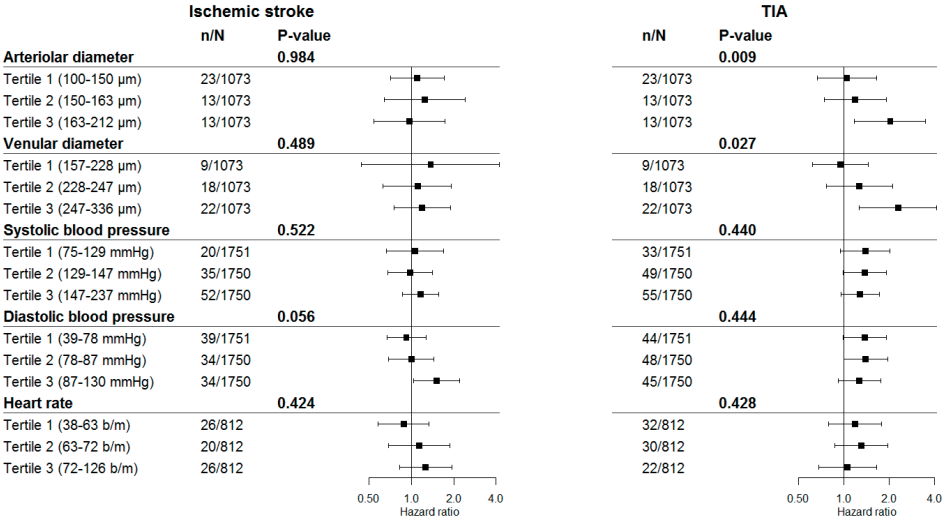

Figure 2. Assessment of effect measure modification between global brain perfusion per SD decrease and various cardiovascular determinants on the risk of TIA and ischemic stroke.
Assessing effect measure modification by stratification and interaction terms between global brain perfusion per SD decrease and markers of microvascular disease (arteriolar and venular retinal diameter), and cardiovascular determinants (systolic and diastolic blood pressure and heart rate). P-value indicates p-value for interaction. Abbreviations: µm = micrometer; mmHg = millimetre of mercury; b/m = beats per minute; n = number of cases; N = number of persons at risk, which may differ per stratified analyses depending on the availability of data within the study population. Cox regression model I: Adjusted for sex, age, study cohort and when assessing retinal vessels, the other retinal vessel was included in the model.

DISCUSSION

In this population-based study, we observed that lower global brain perfusion was associated with a higher risk of TIA but not of ischemic stroke.

Previous studies have assessed the association between global brain perfusion and the risk of TIA or stroke among patient-based samples[27, 28]. A meta-analysis showed that lower brain perfusion is associated with increased risk of ischemic events in patients with symptomatic or asymptomatic carotid stenosis or occlusion[7]. In this meta-analysis, the odds ratio (OR) for stroke was 3.87 (95% CI 1.99-7.48) and for TIA and stroke combined 4.65 (95% CI 2.65-8.14). Interestingly, we found no association of lower brain perfusion with a higher risk of stroke, even when censoring for previous TIA, which is in contrast with these earlier clinical studies. An important explanation for this may be the differences in study population. All of the prior findings were based on patients with carotid artery stenosis, whereas our study population comprised of asymptomatic community-dwelling elderly. A study population comprising of patients with carotid stenosis will have more comorbidity, thereby increasing the risk of developing a TIA or stroke.

Surprisingly, we found that the effect of lower global brain perfusion was more pronounced for TIA than ischemic stroke. This implies that lower global brain perfusion is more likely a hemodynamic risk factor for focal transient ischemia than permanent ischemia. It is known that a reduction in global cerebral blood flow may give focal worsening of an existing neurological deficit in the brain, i.e. after TIA or stroke[29]. However, in people with lower global brain perfusion without history of TIA or stroke, probably the combination of a short disruption of global brain perfusion with impaired autoregulatory vasodilation leads to a TIA, while the presence of cardiovascular risk factors are necessary to cause a longer drop in global brain perfusion sufficient to lead to an ischemic stroke. An explanation could be that within an individual, perfusion in the cerebral arteries and larger arterioles fluctuates with the cardiac cycle[30]. Given a lower mean brain perfusion, these fluctuations resulting in peaks and troughs, may during a trough drop below the critical ischemia point causing a mild and short ischemia in the brain and consequently a TIA, but not a stroke due to a fast recovery from this trough in this brain perfusion cycle[31]. Such a harmful drop in brain perfusion will especially occur when cerebral autoregulation is poor for example by impaired myogenic mechanisms[32]. Indeed, in the analyses in which we stratified by retinal vessel diameters, we found evidence that in participants with wider retinal calibers, the association between global brain perfusion and TIA was stronger, but did not modify the association with ischemic stroke (Figure 2). Myogenic autoregulation is widely distributed over vascular beds including the retina and brain[33]. Autoregulation of the retinal vessels may thus reflect autoregulation in the small brain vessels. If arterioles are already fully vasodilated, they may not be able to widen any further in response to a drop in perfusion. Thus, our findings of more pronounced effects in persons with widened retinal vessels suggest exhausted vasodila-

tory response to be an important contributing factor for TIA in the general population. For ischemic stroke to occur, however, it is likely that more is needed than a drop in global brain perfusion, namely the presence of cardiovascular risk factors. This is supported by our finding that participants with higher diastolic blood pressure show an increased risk for developing ischemic stroke per decrease of global brain perfusion. Another possibility is that a longer drop in global brain perfusion is needed to develop a stroke, for which a lower global brain perfusion in the general population is not a risk factor alone. Although TIA and ischemic stroke share many risk factors, specifically with regard to the role of global brain perfusion our study also suggests the existence of specific etiological differences. Further studies are needed to determine to which factors the lack of effect of global brain perfusion on ischemic stroke in the general population are attributable, for instance collateral circulation in the brain.

Limitations of our study include the following. First, we used single global brain perfusion measurements in relation to the risk of TIA or stroke. We could not perform analyses on repeated measurements of brain perfusion, due to small numbers at this moment. Hence, we could not assess fluctuations in perfusion or display changes of perfusion over a longer period of time. In addition, more advanced imaging techniques such as arterial spin labelling tend to have lower and slightly less variable absolute perfusion measures due to post processing with a single value deconvolution model, compared to phase contrast imaging measures of perfusion, where overestimation of brain perfusion can particularly occur in aged brains due to usage of a max slope model[34]. But such a systematic deviation would not influence obtained relative risks and a larger variability would only lead to dilution of effect estimates. It is important to acknowledge that using our phase-contrast method we obtain a measure of blood flow in major brain arteries which is assumed to represent homogeneous flow in the entire brain under normal conditions. This directly highlights that potentially interesting inhomogeneities of perfusion among different brain regions cannot be taken into account. There may be compensation on other brain regions and these hypo- and hyperperfusion regions may average out in the carotid and basilar arterial flow. Second, the vast majority of our population is of European ancestry, limiting generalizability to other ethnicities. More specifically, it is known that the impact of small vessel and large vessel disease on stroke is different between ethnic groups, where in Caucasians stroke is more often associated with large vessel disease (carotid stenosis) compared to small vessel disease (lacunar infarcts) in Africans and Asians[35]. Further study is needed to confirm that these findings indeed apply to other ethnicities and geographical regions.

Strengths of our study are the population-based setting and the thorough assessment and follow-up for stroke and TIA. Another strength is the amount of stroke and TIA cases, which allowed us to investigate the association with brain perfusion for these outcomes separately. Finally, our cohort includes both women and men, allowing us to investigate a heterogenic

population, although we found no effect modification of the association between brain perfusion and risk of ischemic stroke or TIA by sex in our study (data not shown).

In conclusion, impaired global brain perfusion increases the risk of TIA but not of ischemic stroke in this community-dwelling population. These findings support a role of global brain perfusion as a hemodynamic risk factor for developing a TIA. Further studies are warranted to unravel mechanisms in relation to compensatory mechanisms such as autoregulatory vasodilation and collateral circulation in the brain. Also more detailed regional changes that cannot be detected by phase-contrast imaging are important to take into consideration in order to assess the potential of global brain perfusion as a target for prevention of vascular events.

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Chapter 6
General Discussion

FINDINGS IN PERSPECTIVE AND METHODOLOGICAL CONSIDERATIONS

Neurodegenerative and vascular pathologies interact, which is clinically shown in the frequent co-occurrence of dementia and stroke. Therefore, widespread prevention measures of both dementia and stroke are becoming an inevitable necessity.[1,2] While stroke is an acute disorder with the key clinical feature of sudden onset of a focal neurological deficit, Alzheimer's disease (AD) is a progressive disorder, with blood-based biomarkers becoming increasingly important for the diagnosis and prognosis of AD. In the past, the only validated biomarkers for AD were amyloid and neurofibrillary tangles.[3] I aimed to uncover new promising biomarkers for AD in **chapters 2** and **4** using a population-based approach, with a focus on the role of immunity and inflammation.

I studied the balance between innate versus adaptive immunity in relation to the risk of dementia using blood cell counts in **chapter 2.1** and found that biomarkers reflecting this balance were associated with an increased dementia risk. I also found in **chapter 4.2** that these immune biomarker levels differ in *APOE* haplotype, which remains the risk factor with the largest effect for developing sporadic AD.

IMMUNITY AND DEMENTIA

To support the role of immunity in AD, I tried to triangulate our findings of **chapter 2.1** by performing a two-sample Mendelian Randomization (MR) Study (**chapter 2.2**). This study suggests however that lifelong elevated circulating immune biomarkers are not associated with AD risk or hippocampal volume. So is immunity then really playing a role in AD? Let us have a closer look at the findings in **chapter 2.1**. There I find that especially an increase of platelets over time leads to an increased risk of all-cause dementia, even when censoring for stroke. These estimates are higher for Alzheimer's disease and even higher for vascular dementia as outcomes. Therefore, I expected to find an association between platelets and Alzheimer's disease risk using MR. There are several potential methodological explanations as why I did not find this association with MR as outlined in **chapter 2.2**. However, it could also be that an increase in platelets solely is not causally related to AD, and that a larger immunity disruption is needed to cause AD, such as an additional disruption in the adaptive immune system. Indeed, I find in **chapter 2.2** that CD4 cell counts, important players of the adaptive immune system, show the strongest suggestive association with AD. This notion is supported by the findings in **chapter 2.1** where I show that a doubling in PLR, reflecting an imbalance in platelets and lymphocytes, is also significantly associated with AD and that a doubling of lymphocytes displays a protective effect for AD especially within *APOE* allele 4 carriers.

Several mechanisms could explain these associations. First, the classical vision on the innate immune system that it is merely the immediate mediator of host resistance and inflammation is switching into the notion that it also holds a memory similar to the adaptive immune system.[4] Although primarily a protective response by increasing resistance to reinfection, under predisposing conditions innate memory may endorse human diseases characterized by excessive inflammation.[5] Moreover, emerging evidence suggests that microglia are able to sense inflammatory signaling molecules originating outside the brain.[4,6] Thus, circulating innate immune myeloid cells could participate in the long-lasting dysregulated AD immune response by acting either directly when recruited to brain, or indirectly through the release of soluble mediators.[4,7] Second, regarding adaptive immunity, a study found that genetic ablation of peripheral immune cell populations significantly accelerates amyloid pathogenesis, worsens neuroinflammation, and alters microglial activation state.[8] They show that loss of IgG-producing B cells impairs microglial phagocytosis, thereby exacerbating amyloid deposition. Replacement of IgGs via direct injection or bone marrow transplantation reversed these effects and reduced Aβ pathology. Together, these results support the importance of the adaptive immune system and its interactions with microglia in the pathogenesis of AD.

But what triggers this imbalance in innate and adaptive immunity? A proposed new model suggests that infections of the brain, and infection elsewhere in the body that activates the immune system in the brain could be a potential trigger.[9] Particularly agents capable of producing an ineradicable infection, like herpesviruses, theoretically could trigger chronic neuroinflammation. In **chapter 2.4** I therefore linked herpes simplex virus type 1 (HSV-1) to AD, but found only an association between HSV-1 and risk of dementia within younger individuals. Several other microbes also have been linked to AD, including *Helicobacter pylori (H. pylori)*, which we also investigated **(chapter 2.3**). Similar to HSV-1, I found no association between *H. pylori* and AD risk in the total population, however risk estimates were higher in older participants, possibly due to exposure to more virulent strains in earlier years. Despite the null-findings considering infection and AD within the Rotterdam Study population, it is still plausible that infection leads to the formation of β-amyloid. For some investigators have suggested the "antimicrobial protection hypothesis", arguing that β-amyloid is capable of functioning as a natural antimicrobial peptide that is effective against not only viruses but also bacteria and fungi.[10] However, these smaller, soluble forms (oligomers) of β-amyloid are not only potential antimicrobials, they are also still neurotoxic. These neurotoxic effects typically become apparent only after age 60 years, potentially explaining its conservation throughout evolution for its beneficial effects earlier in life, at times where life expectancy was shorter and infectious diseases were important hazards.[9]

To assess causality of immune mechanisms with AD, several study designs were considered in **chapter 2**, with each having its own strengths and limitations. In **chapters 2.3** and **2.4**, I used the prospective cohort design using a single measurement for the determinant.

Limitations include reverse causation and residual confounding. In contrast to using a Cox regression model, the studies described in **chapters 2.1** and **3.1** again used the prospective cohort design but now using repeated measurements for the determinant using joint models. This statistical model is in particular useful here since the internal covariates (blood cell counts) are intermittently measured (biomarker levels are only known for the specific time points the participant visited the study center), and because they also contain measurement error, their complete path up to any time point t is not fully observed.[11] An important strength of the joint model is thus that we can successfully reconstruct the complete longitudinal history by postulating a suitable mixed-effects model to describe subject-specific time evolutions. The mixed model then accounts for the measurement error problem by postulating that the observed level of the blood cell count measurements equals the true level of the blood cell count measurements plus a random error term. However, since the survival function S depends on the whole history of the true marker levels (M), it is important to obtain a good estimate of M for an accurate estimation of S.[12] Thus, careful examination of data is important since effects could be for instance non-linear, as in **chapters 5.2** and **4.3**. Finally, we performed an MR study (**chapter 2.2**), a design in which genetic variants are used as 'instrumental variables' for a putative causal factor, theoretically minimizing the possible influence of confounding or reverse causality. If we seek to assess whether there is a causal effect of trait A (e.g. immune cells) on outcome B (AD), but not to provide an estimate of a causal effect parameter, then only the three instrumental variable assumptions listed in **Figure 1** are required. To estimate a causal effect parameter, further assumptions are required, namely 1) linearity of the risk factor-outcome association, and 2) the stable unit treatment value assumption (the value of the outcome for each individual depends on the value of the risk factor, and not on the mechanism by which the risk factor was intervened on). MR studies are often compared to randomized controlled trials (RCTs), but the causal effect estimate from an MR study should not be interpreted literally as the expected outcome of an intervention on the risk factor of interest. In almost all cases, we can only make inferences on the direction of the effect from an MR study rather than estimating an effect size.[13]

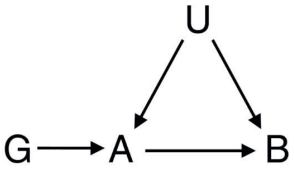


Figure 1. Diagram illustrating causal relationships between genetic variants G, putative causal trait (risk factor) A, putative effect trait (outcome) B, and confounders U necessary for instrumental variable assumptions to be satisfied.[13] The three instrumental variable assumptions are: 1) The set of genetic variants is associated with risk factor A; 2) Each genetic variant is independent of confounders U of the association between A and B; 3) If the risk factors were kept constant, intervention on the genetic variants would not have an effect on the outcome.

IMMUNITY AND STROKE

Besides the role of immunity in dementia, immunity disruption also seems to play a role in stroke development (**chapter 3**). Using a similar approach as in **chapter 2.1**, I studied how immune biomarkers relate to ASCVD risk since stroke shares many risk factors with other cardiovascular diseases although their relative importance varies (**chapter 3.1**).[14] In contrast to **chapter 2.1**, where an increase of platelets leads to the highest dementia risk, I found in **chapter 3.1** that granulocytes are the most important immune biomarkers for ASCVD risk, although platelets were still borderline significantly associated with a higher stroke risk, especially ischemic stroke. These findings suggest that immunity is indeed an overlapping mechanism between dementia and stroke, and that specifically platelets could form the bridge between the two diseases.[15] Not only do platelets play a role in vessel occlusion leading to stroke, they are also important players in inflammation.[16] Moreover, they express the amyloid precursor protein (APP) and display the complete enzymatic machinery to process APP proteins into β-amyloid as mentioned earlier.[17]

In addition to the role of platelets, in **chapter 3.1** I furthermore show that subclinical atherosclerosis as measured by carotid arterial calcifications potentially mediates the role of granulocytes in stroke risk. This suggests immunity-driven atherosclerosis as another important common mechanism between dementia and stroke.[18] Indeed, allelic variants in common genes including *APOE* predispose to both atherosclerosis and dementia.[18] Moreover, I found that innate immunity was linked to larger plaques, whilst the adaptive immunity (lymphocytes) related to smaller plaques and a lower frequency of intraplaque hemorrhage (**chapter 3.2**). I found similar protective effects of lymphocytes on all-cause dementia risk in **chapter 2.1**. Taken together, these results suggest that an imbalance in innate and adaptive immunity does not only play a role in dementia risk, but also in stroke risk through increased vulnerability to carotid atherosclerosis rupture.

Several methodological issues warrant consideration regarding the findings in this chapter. First, the mediation analysis presented in **chapter 3.1** presents large effect sizes, but they are not statistically significant. The assessed association between immunity and calcification is cross-sectional, is prone to measurement error and subject to reverse causality. I similarly used a cross-sectional design in **chapter 3.2**, highlighting that causality cannot be inferred solely based on these results. Second, multiple testing can be an issue because the more inferences are made, the more likely erroneous inferences are to occur. Several statistical techniques have been developed to deal with this issue, which generally require a stricter significance threshold for individual comparisons.[19] An example is the usage of the false discovery rate (FDR) as I have done in **chapter 2.2** when applying MR on multiple exposures or the usage of a Bonferroni correction as I have done in **chapter 4.2**.[19] However, in **chapters 3.1** and **3.2** I similarly perform multiple tests, where I associate multiple immune cells and their derived ratios with multiple outcomes, but I did not adjust for multiple

testing. This is because reducing the type I error for null associations also increases the type II error for those associations that are not null. Therefore, some researchers prefer not making adjustments for multiple comparisons because it will lead to fewer errors of interpretation when the data under evaluation are not random numbers but actual observations on nature, thereby avoiding the possibility of missing important findings.[20]

IMMUNITY: THE CONNECTION BETWEEN DEMENTIA AND STROKE

In **chapter 4.1** I have investigated how plasma β-amyloid and NfL relate to the risk of stroke within the Rotterdam Study and I found that higher levels of β-amyloid 40 were related to higher stroke risk in *APOE* ε4 carriers and within younger participants. Furthermore, higher levels of plasma NfL at baseline were associated with a higher risk of incident stroke in the total population. Interestingly, in **chapter 4.2**, I found that β-amyloid 40 and NfL were elevated in participants with higher immune biomarker levels, especially within *APOE* allele 4 carriers, suggesting that immunity disruption may be the trigger of β-amyloid 40 deposition in the vessel wall. In contrast to β-amyloid, blood NfL concentrations are non-specific, reflecting only underlying neuronal or axonal damage. This further underscores an early role of the immune system in neuronal damage, potentially inducing dementia and stroke.

An important methodological consideration is the validity of immune biomarker measurements in this thesis. The question is whether blood cell counts truly reflect the balance between innate versus adaptive immunity. Indeed, the immune system is extremely complex, and consists of many more components than blood cells. For instance, glial cells, more specifically microglia, are important players as immune cells of the central nervous system. However, there is not a paradigm for studying microglia within the Rotterdam Study. Therefore, I provide suggestions as to which biomarkers could additionally be measured to have a better reflection of the balance between innate and adaptive immunity in the **Future Research** section of the Discussion.

Another important consideration is the relative relevance of the findings in this thesis. For instance, in **chapter 4.2** the innate immunity phenotypes were related to higher Aβ, most strongly with a doubling in GLR leading to a 1.9% higher Aβ42 (95% confidence interval [95% CI] 0.4 to 3.3%) and 3.2% higher Aβ40 (95% CI 2.0 to 4.3%). These estimates, although statistically significant, are not very high. A valid question is therefore to what extent the immune system contributes to the onset of AD, or stroke, compared to other risk factors. The failures of several dementia prevention trials illustrates that identifying strong risk factors as potential targets for intervention is a field-wide problem. These trials have mainly focussed on reducing Aβ. Assuming the innate immune system and consequently the production of Aβ as a physiological process, it could be dangerous to inhibit this system to

prevent or treat AD. Regulating the more downstream effects of Aβ, however, could be more feasible considering the mere detrimental consequences of tau pathogenesis leading to tau-mediated neurodegeneration.[21] Thus, although effect sizes are generally small in this thesis, they do provide directions for further research, coming closer to successful interventions. At the end of this **Findings in perspective and methodological considerations** section, I therefore propose an updated model for AD pathogenesis, adding immunity to the other risk factors.

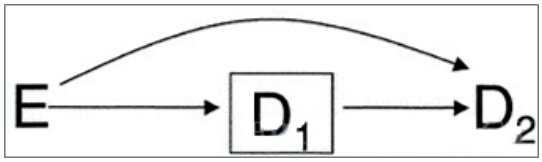
BRAIN PATHOLOGY

Another changing biomarker is tau, which is also assessed using blood-based assays within the Rotterdam Study. Total-tau has been previously assessed in association with dementia risk within the Rotterdam Study, where a J-shaped association was found. Similarly, in **chapter 4.1** we show a J-shaped association between this same biomarker and stroke risk within the Rotterdam Study. Interestingly, I found within **chapter 4.2** only an association between the platelet-to-lymphocyte ratio (PLR) and lower plasma total-tau levels within the same study population. This suggests a less clear role of the immune system in relation to tau, and suggests that tau changes are probably a consequence of β-amyloid abnormalities rather than being directly affected by the immune system. Since the PLR is only associated with higher β-amyloid and NfL within *APOE* allele 4 carriers, whereas these biomarkers are not altered in participants homozygous for *APOE* allele 3 and even lower within *APOE* allele 2 carriers, it could be that a lower plasma total-tau indeed reflects tau abnormalities within the brain when PLR is elevated. The reason that I only find a relation between the PLR and plasma total-tau, and not between the other immune biomarkers, could be because platelets are the primary source of β-amyloid in human blood (~90%)[22], and this secreted peptide is similar to those found in the senile plaques of Alzheimer's patients.[23]

Cognitive impairment is another important biomarker in the progression towards AD. Cognitive functions are mediated by specialized areas of neocortex distributed across the cerebral hemisphere in an orderly arrangement.[24] The cortex of each cerebral hemisphere is a continuous sheet of gray matter, which can be damaged by toxic peptides such as β-amyloid or by loss of neuronal integrity by the phosphorylation of tau. These aging-related processes can lead to cognitive impairment. Telomere length is an important biomarker for such aging-related processes, because telomeres shorten not only with each cell division, but also with aging and oxidative stress, both important factors in cognitive function.[25,26] As such, it has been found that community resident elders with longer telomere length exhibited less decline in global cognitive functioning over 7 years relative to elders with short or medium telomere length.[25] In contrast, another study has found that longer telomeres related to worse cognitive outcomes as CSF amyloid-β and tau increases.[27] In line with these contradictory

findings, I found in **chapter 4.3** that telomere length has a U-shaped association with AD risk, especially within *APOE* allele 4 carriers. However, telomere length seems to be related differently to stroke risk. I performed a preliminary analysis within the Rotterdam Study showing that telomere length is linearly associated with stroke, such that lower telomere length increases stroke risk. It could thus be that shorter telomere length reflects vascular pathology, while longer telomere length reflects more the AD-related brain pathology, the latter either through the upregulation of telomerase[27], or survival bias since shorter telomere length also increases risk of all-cause mortality (**Figure 2**).

a.



b.

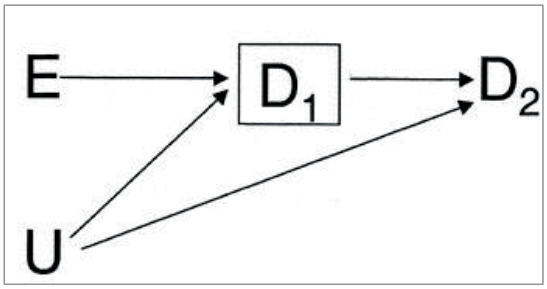


Figure 2a. Effect of telomere length (*E*) on survival (D_1) and dementia (D_2). Suppose the effect of exposure (longer telomere length) on D_1 is protective. Then the hazard at time 2 is the probability of dementia at time 2 among those who survived past time 1, i.e. D_1=0 (square around D_1 indicates this conditioning). The lack of an arrow from *E* to D_2 indicates that, although the exposure *E* has a direct protective effect (decreases the risk of death) at time 1, it has no direct effect on dementia at time 2. Exposure *E* would only have a protective effect on dementia at time 2 when there would be a direct arrow from E to D_2 as now drawn. **Figure 2b.** In the presence of an unmeasured shared cause of death and dementia, then the hazard ratio at time 2 is a biased estimate of the direct effect of exposure on dementia at time 2 from *E* to D_2 through D_1 and *U*. Modified from *Hernán, Miguel A.; Hernández-Díaz, Sonia; Robins, James M. Epidemiology15(5):615-625, September 2004.*

An important methodological issue needs to be taken into consideration, which is selection bias. This form of bias can be an issue in cohort studies, where informative censoring often takes place.[28] Informative censoring occurs when participants are lost to follow-up due to reasons related to the study. **Figure 2b** shows a scenario that is possible in cohort studies, where an unmeasured cause of death and dementia can be present. When I would only include survivors in the analyses, this would lead to a biased estimate for the telomere length – dementia relationship. However, when I would also include the non-survivors or

'censored' participants in the analyses by using their drop-out or mortality event as a study end-point (i.e. not conditioning on D_1), then the issue of informed censoring would be solved by closing the non-causal path from E to D_2 through D_1 and U as shown in **Figure 2**. I have correctly done so in **chapter 4.3**.

BRAIN HEMODYNAMICS

Changes in brain vasculature are one of the earliest changing biomarkers leading to AD progression. Indeed, a multifactorial data-driven analysis using the Alzheimer's Disease Neuroimaging Initiative (ADNI) data suggested that vascular dysregulation is an early step that leads to late-onset AD.[29] These data are supported by a study within the Rotterdam cohort showing that low global brain perfusion precedes cognitive impairment.[30] Since a large proportion of dementias can be prevented by preventing stroke[1], it is important to maximize our understanding of risk factors for stroke, moving beyond atherosclerosis and thromboembolism. While a reduced global brain perfusion is a risk factor for transient ischemic attack (TIA) in the general population (**chapter 5.1**), results in **chapter 5.1** also suggest that a reduced brain perfusion is only a risk factor for ischemic stroke within individuals with elevated diastolic blood pressure. These findings can be translated to a causal pie (**Figure 3**), where a reduction in global brain perfusion is sufficient to cause a TIA in middle- and older aged individuals, while an extra slice (i.e. component cause) is needed for the occurrence of a stroke.

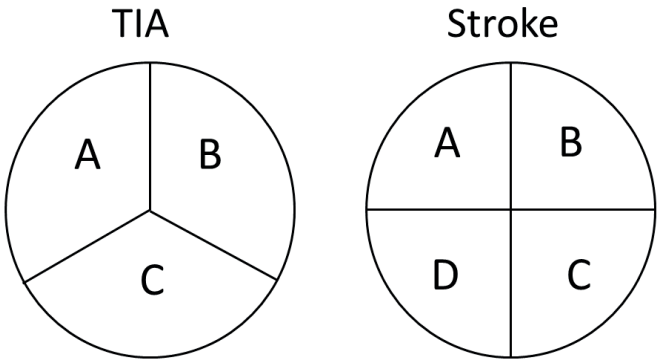


Figure 3. A TIA and stroke consist of a variety of overlapping component causes. Component causes A-C add up to sufficient cause for TIA and component causes A-D add up to sufficient cause for stroke. In this example component A may consist of a variety of cardiovascular risk factors, and component B of genetic risk. The findings from our study suggest that the addition of component cause C, in this case lowered brain perfusion, completes the causal pie for a TIA (i.e. leads to a TIA) whereas another component cause (i.e. D) is still required to lead to a stroke.

Moreover, since a reduced global brain perfusion seems to be a shared risk factor of dementia and stroke, it is of utmost importance to identify individuals that are at increased hemodynamic risk for developing these brain diseases. In **chapter 5.2** I showed that thyroid function acts as a promising determinant to intervene on brain perfusion.

The still hypothetical nature of brain perfusion mechanisms in AD and stroke highlights the impact of several methodological issues that slow the understanding of brain hemodynamic deficits in these brain diseases. First and foremost, the assessment of global brain perfusion using phase-contrast imaging within this population-based setting has limitations in comparison to other methodologies such as arterial spin labelling[31] or radioactive water perfusion measurements using PET.[32] There may be meaningful inhomogeneities of perfusion among brain regions that is not reflected by the average perfusion of the carotid and basilar arteries as measured by phase-contrast imaging. The lack of association between global brain perfusion and ischemic stroke thus remains to be confirmed. Second, I used retinal vessels as proxy for the brain microvasculature. If this can be extrapolated to the entire brain microvasculature remains an open question in the field, but over the years, it has been thought that these structures provide a direct measure for the vascular and neuronal status of the brain.[33] Reduced blood flow following Poiseuille's equation can be produced by altering vessel diameter, reducing viscosity and altering vessel length. I found an inverted U-shaped association between free thyroxine and arteriolar retinal vessel diameter, similar to global brain perfusion in **chapter 5.2**. Therefore, in the context of brain perfusion, I used retinal vessel diameter in this chapter as additional parameter of brain hemodynamics, although the effect is only within the retina and static, whereas with true autoregulation vessel diameter is naturally dynamic. Third, although most ischaemic stroke is due to embolism, there are also other major causes of stroke such as small vessel disease or other arterial diseases.[14] Unfortunately I was not able to further study ischemic stroke subtypes by stroke etiology due to the heterogeneous assessment of etiology within hospitals and due to a lack of power of stroke etiological subtypes.

Taking all these findings including their methodological considerations together, I propose a hypothetical updated Jack model of AD biomarkers to include the role of the immune system in addition to the more established roles of brain vasculature, amyloid PET, CSF β-amyloid and tau in AD (**Figure 4**). This model proposes that immunity is one of the changing biomarkers that leads to AD progression, but its exact position remains to be clarified. In the following sections, I will discuss how future research could shed more light on the position of immunity and inflammation within AD progression and stroke.

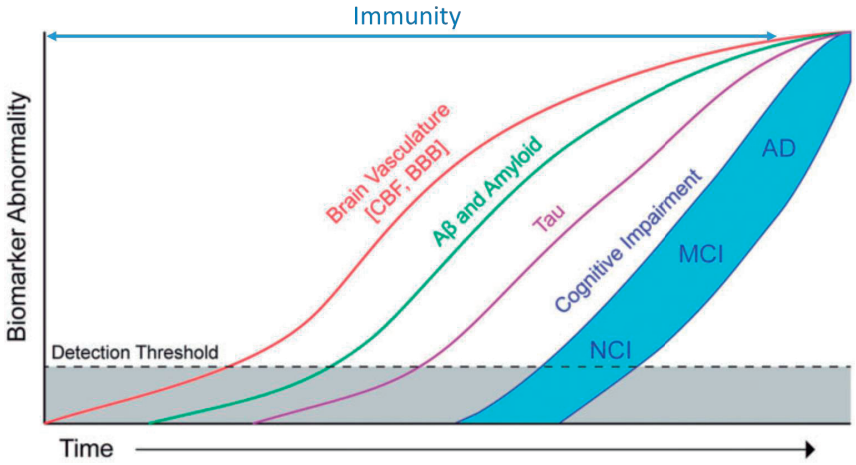
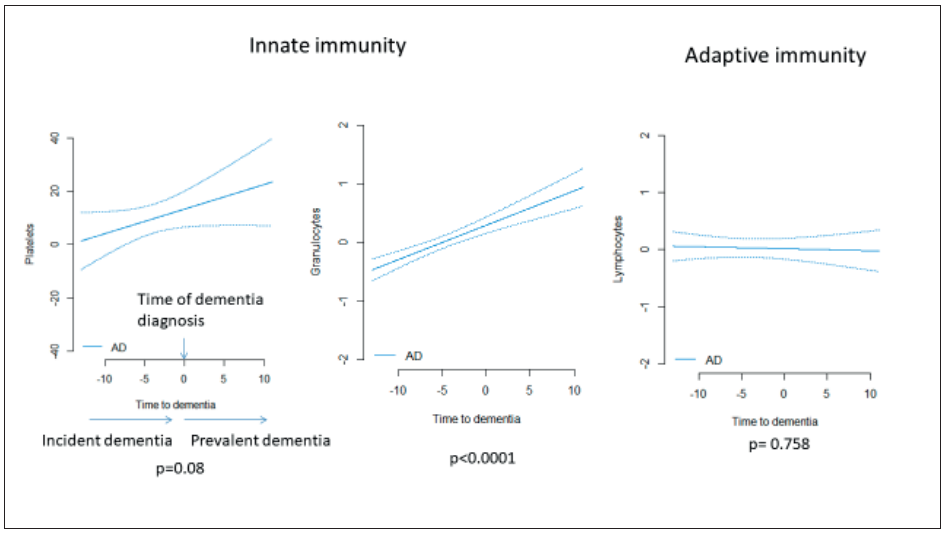


Figure 4. Hypothetical updated Jack model of Alzheimer's disease biomarkers to include the role of immunity. Hypothetical model of AD biomarker changes illustrating that immune changes may contribute to the initial stages of AD pathophysiological progression from NCI to MCI to AD. Abbreviations: AD, Alzheimer's disease; MCI, mild cognitive impairment; NCI, no cognitive impairment. *Modified from Hachinski V, Einhaupl K, Ganten D, et al. Special topic section: linkages among cerebrovascular, cardiovascular, and cognitive disorders: Preventing dementia by preventing stroke: The Berlin Manifesto. Int J Stroke 2019: 1747493019871915.*

FUTURE RESEARCH

To increase our knowledge on the mechanisms underlying dementia and stroke, the most obvious approach is to first reveal the order in which the different components of brain pathology occur, and in particular when inflammation and immunity changes occur. **A statistical technique that can be used for this purpose is an event-based model** (EBM), which is a class of interpretable disease progression models that estimate the timeline of neuropathologic change during AD progression.[34,35] A specific EBM technique, namely discriminative event based model (DEBM), allows inferring the order in which biomarkers for a disease become abnormal based on cross-sectional data, taking into account disease heterogeneity to a larger extent as compared to existing EBM methods.[35] But in order to use this model, it is important to test the assumptions of the DEBM. I propose a method for doing so in **Box** 1. I tested the assumption of a constant increase or decrease of markers over time by constructing a linear mixed model with random slope and intercept and observing if the slopes of the prevalent and incident AD subjects go up or remain constant (**Box 1**). As an example, I show that the assumptions are only met for innate immunity markers. Nevertheless, DEBM provides a useful tool for estimating ordering of biomarker changes provided that the assumptions are met. For this, using more specific adaptive immunity markers that show a constant increase or decrease over time is crucial.



Box 1. Testing the model assumptions of a discriminative event based model.

Conflicting data on the ordering of a disruption in the innate and adaptive immune system in amyloid metabolism have been reported, and remains limited to experimental studies.[36] I aimed to use a novel method for estimating central ordering of events, using the discriminative event-based model applied within the Rotterdam Study.[35] As markers of the innate immunity I used platelet counts and granulocyte counts and for the adaptive immunity we used lymphocyte counts. In order to use this model, the assumption of a constant increase or decrease of immune markers over time must be met. I tested this assumption by building a linear mixed model with non-AD subjects only to predict the effect of age and sex for granulocyte counts (1). Next, I found the residuals in demented subjects by running a model where I substracted the predicted granulocyte counts from model 1 from the raw granulocyte counts. (2). As a last step I would fit the residuals in a linear mixed model with time to dementia as the exposure variable (3). Results for innate and adaptive immunity markers are shown below, showing that the assumptions are only met for innate immunity markers.

Within this thesis, I have mainly used easily obtainable measures of immunity, which allow for repeated measurements in large samples. However, these measures are fairly gross and simplify the complexity of the immune system. This is especially the case for the adaptive immune system, for which I used lymphocyte count as proxy measure. But lymphocytes include natural killer cells, T cells, and B cells, and thus form a heterogeneous group with distinct functions.[37] Of these lymphocyte subsets, T cells seem the most promising target for AD as well as atherosclerosis so far.[38,39] T cells are involved in cell-mediated immunity, with T helper cells producing cytokines that direct the immune response, while cytotoxic T cells produce toxic granules.[40] A first step thus would be **to measure lymphocyte subsets, with a special focus on T cells and further T cell subtyping.** More specifically, in this thesis I suggest in **chapter 4.2** that in particular CD4 T cells are worthwhile exploring further. A recent study showed that CD4 T cells play an important role in microglia maturation which

in turn affects neuronal synapse maturation[41], and another study showed that plaques from symptomatic patients (recent stroke or transient ischemic attack) were characterized by a distinct subset of $CD4^+$ T cells.[39] The old-fashioned notion that the brain is cut off from the peripheral immune system by the blood-brain-barrier (immune privileged) is being reconsidered to the idea that there is truly immune surveillance of the brain.[42] There is promising evidence that temporarily reducing immunosuppressive mechanisms helps release effector T cell activity[43], and at the same time, maintains a sufficient number of anti-inflammatory cells in the periphery.[44] This allows trafficking of inflammation-resolving cells to the sites of brain pathology, in which they play an essential role.[45]

As mentioned earlier, also the measures used for the innate immune system could be expanded. For instance, translocator protein 18 kDa is a marker of activated glial cell response and could be measured using positron emission tomography in a population-based setting.[46-49] **Measuring markers for glial cells that could be detected by imaging therefore may serve as a solution for measuring innate immunity in the CNS.**

In addition to targeting T cells and glial cells, the antimicrobial hypothesis of AD poses that long-lasting infections lead to β-amyloid accumulation, so another approach is to target infections or pathogens to prevent chronic neuroinflammation. Another rationale for this is that there has been a decline in dementia incidence across Western high-income countries.[50] This decline could partly be explained by favorable trends in vascular risk factors. Concomitantly, there has been a decline of mortality by infectious diseases during the 20th century, which could be attributed to improved socio-economic indicators, or use of antibiotics.[51] There has been one randomized controlled trial to study the effect of antibiotics on the progressive decline of cognitive function in patients with AD. Combination antimicrobial therapy with doxycycline and rifampicin significantly reduced cognitive worsening after 6 months but not after 12 months.[52] Future researchers could **investigate the hypothesis that certain antibiotics have a protective effect on the development of dementia**.

To further reveal the order in which the different components of brain pathology occur, and in particular the position of inflammation and immunity changes, is by studying the relation between brain circulation and the immune system. Within the Rotterdam Study, there are ongoing efforts to add arterial spin labelling as a noninvasive measurement of regional cerebral blood flow (CBF).[53] The possibility of **taking these regional variations in CBF into account will allow for studying CBF physiology in relation to immunity and inflammation** within a population-based setting. In addition, it facilitates disentangling the effect of regional CBF on brain outcomes and the determinants that cause regional CBF changes.[54]

Eventually, the goal is to prevent dementia and stroke by understanding pathophysiology and determining clinical relevance of newly discovered pathways, such as the role of immunity and inflammation. This requires a **close collaboration between dementia and**

stroke researchers, epidemiologists, neuroscientists, infection & immunity experts and geneticists. This is crucial for facilitating translational research.

CONCLUSION

In this thesis, I have shown that immunity and inflammation play a crucial role in dementia and stroke. Furthermore, I describe how future research can build upon this work. The prevention of dementia and stroke can be boosted by enhancing our understanding of the order in which different components of brain pathology and hemodynamic changes occur. In particular, the position of immunity and inflammation changes within this cascade warrants further research.

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Chapter 7
Summary & Samenvatting

SUMMARY

The etiological similarity between dementia and stroke was already suggested in Alois Alzheimer's first major article on dementia, describing that those cases of atrophy without recognizable infarcts were due to microscopic infarcts. The similarity between dementia and stroke has been further confirmed by the evidence of substantial drops of dementia in high-income countries where stroke incidence and mortality has declined substantially. But what the exact underlying mechanism is of these two diseases is yet unknown. In this thesis, I examine immunity and inflammation as potential common mechanisms connecting dementia and stroke.

In the first part of this thesis, I demonstrate the role of an imbalance in the immune system towards innate immunity in the pathogenesis of dementia by showing that an increase of components of innate immunity increase dementia risk and an increase of components of adaptive immunity is protective for dementia risk in the general population (**chapter 2.1**). In **chapter 2.2** I did not find evidence to support associations of circulating biomarkers of immunity and inflammation with Alzheimer's disease (AD) and hippocampal volume, but CD4 count, an important player of adaptive immunity, showed the strongest suggestive association with AD. In **chapters 2.3** and **2.4** I found no evidence of *Helicobacter pylori* or herpes simplex type 1 infections to be associated with dementia risk in the general population.

Focussing next on the role of immunity and inflammation in stroke, I show in **chapter 3.1** that an increased granulocyte count reflecting innate immunity was associated with a higher risk of atherosclerotic cardiovascular disease (ASCVD) in the general population. Moreover, higher levels of granulocytes were associated with larger volumes of arterial calcification, with a mediation analysis suggesting that arterial calcifications may explain a proportion of the link between granulocytes and ASCVD. A causal relationship is supported by effects of granulocytes and other immune markers on plaque thickness and plaque components in **chapter 3.2**, suggesting that an imbalance in innate and adaptive immunity may play a role in the vulnerability of the carotid atherosclerotic plaque.

Chapter 4.1 further connects dementia and stroke by finding that participants with higher total-tau and neurofilament light chain at baseline as markers of AD-related brain pathology had a higher risk of stroke. In addition, higher levels of plasma amyloid-β (Aβ) 40 were associated with an increased stroke risk in *APOE-ε4* carriers only. Supporting the role of accumulating brain pathology from an immunity and inflammation perspective in the etiology of dementia and stroke, I found in **chapter 4.2** that higher levels of immunity markers were associated with higher Aβ in plasma. I further show that participants with a higher genetic predisposition to AD had higher immunity markers, where these effects were mainly driven by *APOE*-ε4. In contrast to the more traditional markers of AD-related brain pathology, I also studied telomere length as a promising marker of brain pathology in

chapter 4.3, where I found a U-shaped association between telomere length and risk of AD in the general population and even more strongly so in *APOE*-ε4 carriers.

In **chapter 5**, I shift focus to brain hemodynamics. I found in **chapter 5.1** that lower global brain perfusion was associated with a higher risk of transient ischemic attack, but not with the risk of ischemic stroke, in the general population. This association may be modified by wider arteriolar and venular retinal calibers for transient ischemic attack and elevated blood pressure for ischemic stroke. To identify a potentially amendable determinant to intervene on global brain perfusion, we studied thyroid function in **chapter 5.2** and we found that global brain perfusion was lower in middle-aged and elderly with lower as well as higher FT4 levels. Furthermore, arteriolar retinal vessel diameters were narrower in elderly with higher TSH and TPO levels.

To conclude, I discuss in **chapter 6** these main findings where I have shown that immunity and inflammation play a crucial role in dementia and stroke in light of published literature, and provide methodological considerations. Furthermore, I describe how future research can build upon this work by proposing that the prevention of dementia and stroke can be boosted by enhancing our understanding of the order in which different components of brain pathology and hemodynamic changes occur. Also the position of immunity and inflammatory changes within this cascade are important questions to be answered for future researchers.

SAMENVATTING

De etiologische gelijkenis tussen dementie en hersenberoertes kwam al naar voren in Alois Alzheimer's eerste grote artikel over dementie, waarin hij beschrijft dat patiënten met hersenatrofie zonder duidelijke infarcten waarschijnlijk microscopische infarcten hebben. De gelijkenis tussen dementie en hersenberoertes kwam verder naar voren bij de zichtbare afname van dementie in welvarende landen waar de incidentie van hersenberoertes en mortaliteit substantieel is afgenomen. Wat echter het exacte onderliggende mechanisme is van deze twee fascinerende aandoeningen is nog onduidelijk. In dit proefschrift beschouw ik het immuunsysteem en inflammatie als potentiële overlappende mechanismen die dementie en hersenberoertes kunnen verbinden.

In het eerste deel van dit proefschrift demonstreer ik de rol van een disbalans in het immuunsysteem richting het aangeboren immuunsysteem in de pathogenese van dementie door te laten zien dat een toename van componenten van het aangeboren immuunsysteem het risico op dementie verhogen terwijl een toename van componenten van het aangeleerde immuunsysteem beschermend is tegen het krijgen van dementie (**hoofdstuk 2.1**). In **hoofdstuk 2.2** heb ik geen bewijs gevonden die het verband tussen het immuunsysteem en Alzheimer ondersteunen, maar CD4 cellen, belangrijke spelers van het aangeleerde immuunsysteem, laten de sterkste suggestieve associatie zien met Alzheimer. Kijkend naar de rol van infectieziekten in het ontstaan van dementie, vond ik in **hoofdstukken 2.3** en **2.4** geen associatie tussen de maagbacterie *Helicobacter pylori* of het virus herpes simplex type 1 en dementie in de normale bevolking.

De focus verleggend naar de rol van het immuunsysteem en inflammatie in hersenberoertes, laat ik in **hoofdstuk 3.1** zien dat een toename van granulocyten, die het aangeboren immuunsysteem weerspiegelen, geassocieerd is met een hoger risico op het krijgen van cardiovasculaire aandoeningen. Via een mediatie analyse laat ik zien dat dit verband mogelijk voor een groot deel door de vorming van aderverkalking wordt veroorzaakt. Een causaal verband wordt ondersteund door te kijken naar de effecten van onder andere granulocyten op de dikte van aderverkalking en losse componenten van aderverkalking in **hoofdstuk 3.2**. Deze resultaten suggereren dat een disbalans in het aangeboren en aangeleerde immuunsysteem een rol zouden kunnen spelen in de kwetsbaarheid van aderverkalking om te scheuren.

Hoofdstuk 4.1 legt verder de verbinding tussen dementie en hersenberoertes door de bevinding dat mensen met meer total-tau en neurofilament light chain (biomarkers van Alzheimer-gerelateerde hersenpathologie) een hoger risico hebben op het ontwikkelen van hersenberoertes. Daarnaast zijn hogere spiegels van plasma amyloid-β (Aβ) 40 geassocieerd met een verhoogd risico op het krijgen van hersenberoertes in *APOE-ε4* dragers. Kijkend vanuit het perspectief van het immuunsysteem, heb ik verder in **hoofdstuk 4.2** gevonden dat hogere immuun- en inflammatiewaardes geassocieerd zijn met hogere Aβ in plasma. Verder hebben mensen met een verhoogd genetisch risico op het ontwikkelen van Alzheimer

ook verhoogde immuun- en inflammatiewaardes. Deze effecten worden vooral gedreven door *APOE*-ε4. In tegenstelling tot de meer traditionele markers van Alzheimer-gerelateerde breinpathologie heb ik ook telomeerlengte bestudeerd als een potentiële biomarker in **hoofdstuk 4.3**, waarbij ik heb gevonden dat er een U-vormige associatie is tussen telomeerlengte en het risico op het ontwikkelen van Alzheimer, waarbij deze associatie nog sterker is in *APOE*-ε4 dragers.

In **hoofdstuk 5** verleg ik de aandacht naar breinhemodynamica. Ik heb in **hoofdstuk 5.1** gevonden dat een lagere algehele breinperfusie geassocieerd is met een hoger risico op het krijgen van een transient ischemic attack, ofwel een TIA, maar dat het risico niet verhoogd is op het krijgen van een ischemische hersenberoerte. Deze associatie werd gemodificeerd door wijdere retinale arteriolen en venulen voor het krijgen van een TIA en door een hogere bloeddruk voor het krijgen van een ischemische hersenberoerte. Om een potentiële modificeerbare determinant te vinden om algehele breinperfusie te beïnvloeden, hebben we gekeken naar schildklierfunctie in **hoofdstuk 5.2** en heb ik gevonden dat de algehele breinperfusie lager was in ouderen met lagere alsook hogere FT4 spiegels. Verder waren de retinale arteriolen ook smaller in ouderen met hogere TSH en TPO spiegels.

Tot slot bediscussieer ik in **hoofdstuk 6** de bevindingen waarin ik laat zien dat het immuunsysteem en inflammatie een cruciale rol spelen in de ontwikkeling van dementie en hersenberoertes met inachtneming van de gepubliceerde literatuur en bespreek ik methodologische overwegingen en factoren. Ik beschrijf verder hoe toekomstig onderzoek kan bouwen op dit werk door voor te stellen dat de preventie van dementie en hersenberoertes een boost kan krijgen door te bestuderen wat de volgorde is van het veranderen van verschillende componenten van brein pathologie en hemodynamica. Wat de positie is van immuunsysteem- en inflammatieveranderingen in deze cascade is een cruciale vraag om door toekomstige onderzoekers beantwoord te worden.

Chapter 8
Appendices

PHD PORTFOLIO

Name PhD student: L. Fani **Erasmus MC Department: Epidemiology** **Research School: Netherlands Institute for Health Sciences** (NIHES)	**PhD period: February 2017 – July 2020** **Supervisors: Professors M.A. Ikram and M.K. Ikram**

1. PhD training	Year	ECTS
General courses		
Master of Science in Clinical Epidemiology (NIHES)	2017-2018	70
R course	2017	1.8
Scientific integrity (Erasmus MC)	2017	0.3
ESP62 Markers and Prediction Research (NIHES)	2019	0.7
Seminars and workshops		
Departmental journal club	2017-2020	2.0
Departmental seminars (Epidemiology & Neurology)	2017-2020	4.0
Chair of the seminar committee	2017-2019	1.0
8th Mix & Match meeting of Alzheimer Nederland	2017	0.5
2de gezamenlijke wetenschappelijke vergadering van de Neurovasculaire Werkgroep en het Neurovasculair Genootschap	2018	0.5
International conferences		
The 8th International Conference of The International Society of Vascular Behavioural and Cognitive Disorders (VASCOG)**. Amsterdam, NL**	2016	1.0
4th European Stroke Organisation Conference (ESOC)**. Gothenburg, Sweden**	2018	2.0
9th International Conference of The International Society of Vascular Behavioural and Cognitive Disorders. Hong Kong	2018	2.0
5th European Stroke Organisation Conference (ESOC)**. Milan, Italy**	2019	2.0
Virtual Alzheimer's Association International Conference (AAIC)	2020	1.0
Research visits		
CoSTREAM consortium meeting. Cambridge, UK	2017	1.0
Institute for Stroke and Dementia Research (ISD) **- LMU Klinikum, Ludwig-Maximilians-University in Munich, Germany**	2020	

2. Teaching	Year	ECTS
Teaching assistance		
SPSS and/or R computer practicals of the one-week Biostatistical Methods I	2019	0.5
ESP65 Computer Practical of Practice of Epidemiologic Analysis (NIHES)	2019	0.5

Project supervision		
Master's project: Vitamin D Status and Risk of Stroke	2019	1.5
Master's project: HSV1 and risk of dementia	2020	1.5
3. Other activities		
Peer-review	2018-2020	2.5
Scan appraisal for incidental findings in population imaging	2018-2020	2.0
Stroke data collection of the Rotterdam Study	2017-2020	4.0
Dementia screening with structured interview (CAMCOG/ CAMDEX)	2017-2020	1.5
Physician at ERGO centre performing exit interview	2017-2020	3.0

*1 ECTS (European Credit Transfer System) equals a workload of 28 hours

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ABOUT THE AUTHOR

Lana Fani was born on the 15th of June 1991 in Capelle aan den IJssel, the Netherlands. She grew up in a loving family of Syrian origin, in Rotterdam, and followed bilingual (English A1 higher level) education at the Wolfert van Borselen scholengroep. During her final years at high school she was introduced to the brain by following a Pre-University Class at the University of Leiden on Psychology, after which she started studying Medicine at the Erasmus University Rotterdam. She became an active member of the International Federation of Medical Students' Assocations (IFMSA) by joining the National Board of IFMSA-NL, while also working as a translator between doctors and patients for the Arabic (Middle Eastern) language at Erasmus MC. Her career in research started simultaneously early during her studies by working as a student-assistant at the department of Neurosurgery, which resulted in a first author publication in a peer-reviewed international medical journal. Her second first-author publication during her Bachelor followed soon after performing a systematic review at the department of Pediatric Endocrinology. Due to her increasing interest in research and the brain she followed the Neuroscience Research Master's program at Erasmus MC. After that, she completed her clinical rotations, and started as a PhD candidate at the Department of Epidemiology at Erasmus MC. During that period, she obtained a Research Master's degree in Clinical Epidemiology at the Netherlands Institute of Health Sciences. Eager to learn new research methods and to work in different environments, she performed part of the work of her thesis at the Ludwig-Maximilians-Universität in Munich (Germany) at the Institute for Stroke and Dementia Research, supported by a personal fellowship of the Dutch Alzheimer Foundation. During the completion of her thesis, she started working as an Internal Medicine Physician at Ikazia Hospital Rotterdam. Lana enjoys playing the piano in her free time. Her goal is to become a Neurologist, combining clinical and research activities in an academic setting.

EPILOGUE

The work in this thesis could not have been made possible without the guidance and support of many inspiring people.

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